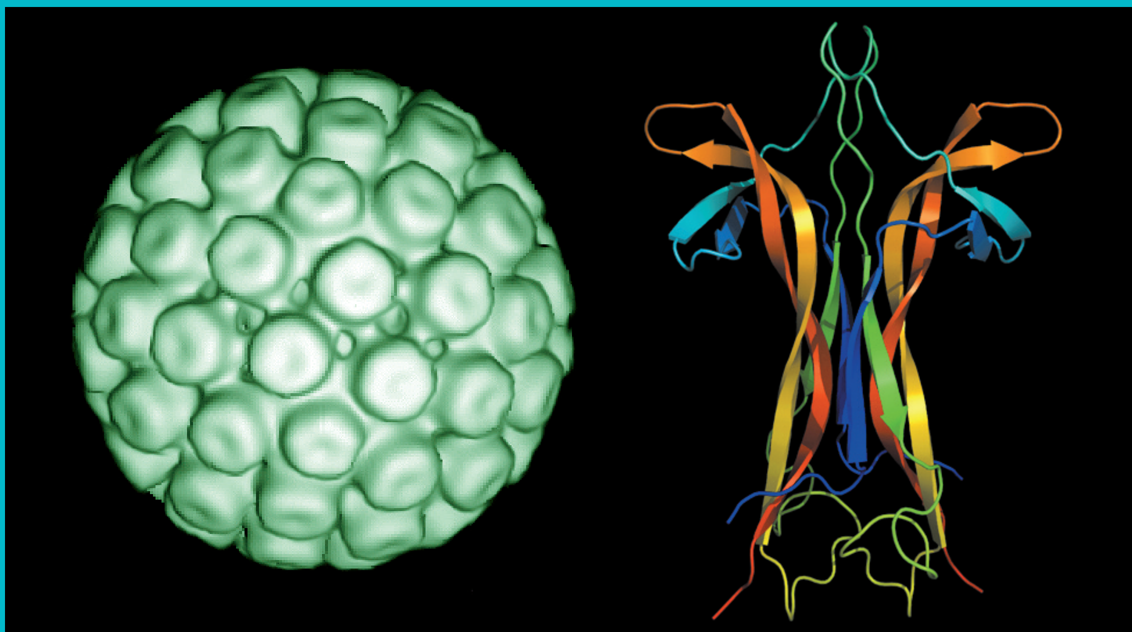
Fondazione Telethon

XVII Scientific Convention

March 11-13, 2013

Riva del Garda - Palazzo dei Congressi



Provincia Autonoma di Trento

Fondazione Telethon

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RIVA DEL GARDA (TN)

PROVINCIA
AUTONOMA
DI TRENTO

The cover of this Book of Abstracts is in honour of Renato Dulbecco and Rita Levi Montalcini, whose scientific discoveries and personal commitment keep inspiring the Telethon scientific community.

Front cover: **Nobel inspirations**
Left: Surface shaded three-dimensional image reconstruction of the Simian Virus 40 (SV40). Courtesy by Prof. Timothy S. Baker, University of California, San Diego, La Jolla, CA, USA.
Renato Dulbecco (Catanzaro, 1914 - La Jolla, 2012) was awarded together with David Baltimore and Howard Temin the Nobel Prize in Physiology or Medicine in 1975, for their discoveries concerning "the interaction between tumour viruses and the genetic material of the cell".
Right: Crystal Structure of the Nerve Growth Factor (NGF). Courtesy of Prof. Antonino Cattaneo, Scuola Normale Superiore, Pisa.
Rita Levi-Montalcini (Turin, 1909 – Rome, 2012) was awarded together with Stanley Cohen the Nobel Prize in Physiology or Medicine in 1986, for their discoveries of the Nerve Growth Factor (NGF) and the Epidermal Growth Factor (EGF), respectively.

Back cover: **Rita Levi-Montalcini and Renato Dulbecco** at an award ceremony, 1987. LaPresse.

Renato Dulbecco and Telethon
Renato Dulbecco chaired the Telethon Scientific Committee from 1991 to 1995, of which he was Honorary President until his death in 2012. His contribution to the activities of the Fondazione Telethon was also significant as a result of his participation in the work of the first Scientific Advisory Board; it was the elaboration of this strategic advisory body which resulted in the creation of the Telethon Career Projects, which evolved into the Dulbecco Telethon Institute. In 1999 Dulbecco participated in the Sanremo Festival, from which he donated his entire honorarium towards the development of an initiative that would allow young researchers to conduct, fully independently, their scientific careers in Italy. Since then, the Dulbecco Telethon Institute (DTI) has engaged with 30 laboratories in Italy and has allowed more than 370 researchers among which are "Telethon scientists", associates, and fellowship holders to carry out their activities, enriching with their talents the community of Italian scientists engaged in research into genetic diseases.

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Provincia Autonoma di Trento

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SCIENTIFIC PROGRAM

Monday, 11th March 2013

10.00 – 14.00 *Registration and poster setting up*

14.15 – 14.30 **Welcome** ***Sala 1000***

14.30 – 15.00 **Opening address**
Lucia Monaco (Fondazione Telethon)

15.00 – 15.30 **LECTURE**

An introduction to the International Rare Diseases Research Consortium (IRDiRC)
Irene Norstedt (European Commission, Brussels, Belgium) *(Talk 1)*

15.30 – 17.00 **PLENARY SESSION 1** – **Genes and mechanisms of Intellectual Disabilities: paving the way for potential therapies**

Chairpersons: Han Brunner, Maria Passafaro

Intellectual Disability: lessons from 500 diagnostic exomes
Han Brunner (Nijmegen, The Netherlands) *(Talk 2)*

The X-linked Intellectual Disability protein TSPAN7 regulates excitatory synapse development and AMPAR trafficking
Maria Passafaro (Milan, Italy) *(Talk 3)*

FMR1 expression and FMRP function as possible targets of pharmacotherapy in the Fragile X Syndrome
Giovanni Neri (Rome, Italy) *(Talk 4)*

Molecular bases and in vitro modeling of Cdkl5 dependent infantile neurological disorders
Vania Broccoli (Milan, Italy) *(Talk 5)*

17.00 – 17.30 *Coffee break*

17.30 – 19.00 **POSTER SESSION 1**

19.00 – 20.00 **O.Ma.R. Journalism Award** ***Sala 1000***

Organized by the *Osservatorio Malattie Rare*, *Fondazione Telethon* and *Orphanet Italia* with the collaboration of the *National Rare Diseases Centre (CNMR)*
http://www.premiomalattierare.it/

20.00 – 21.00 *Welcome cocktail*

Tuesday, 12th March 2013

08.30 – 09.00 *Registration and poster setting up*

09.00 – 10.30 **PLENARY SESSION 2 – Therapeutic strategies for Mendelian disorders** ***Sala 1000***

Chairpersons: Luis Galietta, Ennio Ongini (Nicox - Milan, Italy - and Rare Partners)

Pharmacological strategies to rescue chloride transport in Cystic Fibrosis
Luis Galietta (Genoa, Italy) *(Talk 6)*

Exploiting artificial nucleases for targeted transgene integration in human Hematopoietic Stem Cells and induced Pluripotent Stem Cells derived from normal donors and SCID-X1 patients
Angelo Lombardo (Milan, Italy) *(Talk 7)*

High Content Screening Facility at TIGEM: pharmacological approaches to treat rare genetic diseases
Diego Medina (Naples, Italy) *(Talk 8)*

A computational strategy to discover pharmacological chaperones with therapeutic potential for Retinitis Pigmentosa
Francesca Fanelli (Modena, Italy) *(Talk 9)*

10.30 – 11.00 *Coffee break*

11.00 – 12.30 **POSTER SESSION 2**

12.30 – 13.00 **PICTURE OF TELETHON SCIENTISTS**
taken by Filippo Sbalchiero — *gathering point: registration desk*

13.00 – 14.00 *Buffet Lunch*

14.00 – 15.00 **KEYNOTE LECTURE** — ***Sala 1000***

TIGEM goes translational
Andrea Ballabio (Naples, Italy) *(Talk 10)*

Imagining new therapies for patients with Rare Diseases: Shire HGT and TIGEM translate the future
Arthur Tzianabos (Shire - Lexington, MA, USA) *(Talk 11)*

15.00 – 16.00 **PLENARY SESSION 3 – Advances in clinical trials**

Chairpersons: Robertson Parkman (Los Angeles, CA, USA), Luigi Naldini (Milan, Italy)

The development of Givinostat for the treatment of Duchenne Muscular Dystrophy: from the animal to the clinic
Paolo Bettica (Italfarmaco – Cinisello Balsamo, Italy), Eugenio Mercuri (Rome, Italy) *(Talk 12)*

Phase I/II clinical trial of Hematopoietic Stem Cell gene therapy for the treatment of Metachromatic Leukodystrophy
Alessandra Biffi (Milan, Italy) *(Talk 13)*

Clinical trial of Hematopoietic Stem Cell gene therapy for Wiskott-Aldrich Syndrome
Alessandro Aiuti (Milan, Italy) *(Talk 14)*

16.00 – 17.00 **ROUND TABLE – Navigating the Valley of Death**
An interactive session engaging all Telethon scientists in tackling the hurdles of translating laboratory research to the clinic

Moderators: Robertson Parkman (Los Angeles, CA, USA), Helen Heslop (Houston, TX, USA)

Speakers: Luigi Naldini (Milan), Alberto Auricchio (Naples), Grazia Valsecchi (University of Milano-Bicocca, Milan, Italy), Alessandro Aiuti (Milan), Paolo Bettica (Italfarmaco – Cinisello Balsamo, Italy)

17.00 – 17.30 *Coffee break*

17.30 – 19.00 **POSTER SESSION 3**

19.00 – 20.00 **SPECIAL EVENT** — ***Sala 300***

The Executive Director of the American Society for Cell Biology meets young investigators

Stefano Bertuzzi (ASCB - Bethesda, MD, USA)

Wine and Cheese

Wednesday, 13th March 2013

09.00 – 10.30 **PARALLEL SESSIONS A-B**

A- Cardiomyopathy - failure and success — ***Sala 1000***

Chairpersons: Daniel Garry, Silvia Priori

Emerging therapies for DMD Cardiomyopathy
Daniel Garry (Minneapolis, MN, USA) *(Talk 15)*

Channelopathies: moving toward gene therapy
Silvia Priori (Pavia, Italy) *(Talk 16)*

Melusin gene therapy: an innovative approach to prevent Familial Cardiomyopathies
Guido Tarone (Torino, Italy) *(Talk 17)*

Inherited Cardiomyopathies: from phenotype-based to genetic-based nosology
Eloisa Arbustini (Pavia, Italy) *(Talk 18)*

B- Decoding an engineering marvel: insights from genetic Renal Diseases

Chairpersons: Gregory Germino, Antonella De Matteis — ***Sala 300***

On the edge of glory? Polycystic Kidney Disease research as a model for moving from bench to bedside
Gregory Germino (Bethesda, MD, USA) *(Talk 19)*

Uromodulin and chronic diseases of the kidney
Luca Rampoldi (Milan, Italy) *(Talk 20)*

Cell biology and pharmacology of Lowe Syndrome
Antonella De Matteis (Naples, Italy) *(Talk 21)*

CLC-5, an endosomal chloride – proton exchanger mutated in Dent's Disease: a bio-physical perspective
Michael Pusch (Genoa, Italy) *(Talk 22)*

10.30 – 11.00 *Coffee break*

11.00 – 13.00 **PARALLEL SESSIONS C-D**

C- Trial readiness in Neuromuscular Diseases — ***Sala 1000***

Chairpersons: Jeffrey Chamberlain, Rossella Tupler

Therapeutic potential of AAV-microdystrophin vectors for gene therapy of Duchenne Muscular Dystrophy
Jeffrey Chamberlain (Seattle, WA, USA) *(Talk 23)*

Facioscapulohumeral Muscular Dystrophy: a walk on the dark side of the (epi)genome
Davide Gabellini (Milan, Italy) *(Talk 24)*

From the bench to the clinic: what we have learnt from the Italian National Registry for Facioscapulohumeral Muscular Dystrophy
Rossella Tupler (Modena, Italy) *(Talk 25)*

Trial readiness in Peripheral Neuropathies: the Charcot-Marie-Tooth Disease pathway
Davide Pareyson (Milan, Italy) *(Talk 26)*

Trial readiness in Peripheral Neuropathies: developing a unifying treatment strategy
Carla Taveggia (Milan, Italy) *(Talk 27)*

D- Blood Disorders: from genetics to genetic therapies — ***Sala 300***

Chairpersons: Punam Malik, Giuliana Ferrari

Mutations in the 5'UTR of ANKRD26 result in a "new" form of Inherited Thrombocytopenia that predisposes to Leukemia and is characterized by the presence in platelets and megakaryocytes of a "new" cell structure
Carlo Balduini (Pavia, Italy) *(Talk 28)*

Hepcidin at the crossroad between Hemochromatosis and genetic Iron Deficiency
Clara Camaschella (Milan, Italy) *(Talk 29)*

RNA-based therapeutic approaches for Blood Coagulation Factor Deficiencies caused by splicing mutations
Mirko Pinotti (Ferrara, Italy) *(Talk 30)*

Gene therapy for non-lethal disorders: the paradigm of Beta-Thalassemia
Giuliana Ferrari (Milan, Italy) *(Talk 31)*

Genetic therapy for Hemoglobinopathies: from the bench to the bedside
Punam Malik (Cincinnati, OH, USA) *(Talk 32)*

13.00 – 13.30 **LATE BREAKING NEWS**

13.30 – 14.00 **POSTER PRIZE AWARD AND CLOSING REMARKS**

Monday, 11th March 2013

10.00 – 13.30 *Registrazione dei partecipanti e ritiro cuffie per traduzione simultanea*

14.15 – 15.30 **SESSIONE PLENARIA (Sala 1000) – Ricercatori e rappresentanti delle Associazioni Amiche insieme**

Apertura dei lavori
Lucia Monaco, Direttore Scientifico – Fondazione Telethon

LECTURE – An introduction to the International Rare Diseases Research Consortium (IRDiRC)
Irene Norstedt - Deputy Head of Unit Personalised Medicine, Health Research Directorate, DG Research and Innovation, European Commission, Bruxelles, Belgio

15.30 – 16.00 *Coffee break*

16.00 – 18.00 **WORKSHOP 1 (Sala 300) - La ricerca farmacologica: prospettive per la cura delle malattie genetiche**

Moderatori: Fabrizio Seidita, Presidente Associazione Italiana Glicogenosi
Francesca Sofia, Fondazione Telethon

Intervento introduttivo di Michael Caplan, Yale University School of Medicine, Presidente del Consiglio d'Indirizzo Scientifico di Telethon

La lunga strada della ricerca: il caso del rene policistico
Alessandra Boletta – Associate Telethon Scientist, DIBIT San Raffaele, Milano

Dallo studio dell'emofilia allo sviluppo di piccoli RNA terapeutici per le malattie genetiche
Mirko Pinotti, Università di Ferrara

Sviluppo informatico di agenti terapeutici: l'esempio della retinite pigmentosa
Francesca Fanelli – Associate Telethon Scientist, Università di Modena e Reggio Emilia

Prospettive della ricerca farmacologica sulle disabilità intellettive
Patrizia D'Adamo – Associate Telethon Scientist, Università Vita-Salute San Raffaele Milano

Discussione con la platea

18.00 – 19.00 **SESSIONE POSTER 1: Incontro con i Ricercatori**

19.00 – 20.00 **Premio Nazionale Giornalistico O.Ma.R. dedicato alle malattie e tumori rari (Sala 1000)**

20.00 – 21.00 *Cocktail di benvenuto*

Tuesday, 12th March 2013

08.30 – 11.00 **WORKSHOP 2 (sala 300) - Costruire la piattaforma clinica per la sperimentazione di nuove terapie per le malattie genetiche**

Moderatori: Eloisa Arbustini, Università di Pavia - IRCCS Fondazione Policlinico San Matteo
Fabio Ciceri, Ospedale San Raffaele Milano

Piattaforma di ricerca clinica per la terapia cellulare avanzata
Fabio Ciceri, Ospedale San Raffaele, Milano

Un registro dei pazienti al servizio dei pazienti: genetica, diagnosi e ricerca per la cura della distrofia facio-scapolo-omerale
Rossella Tupler, Università di Modena e Reggio Emilia

Prepararsi alla sperimentazione clinica: l'esperienza del network italiano per la malattia di Charcot Marie Tooth
Davide Pareyson, Fondazione IRCCS Istituto Neurologico Carlo Besta

Dalla genetica alla terapia: il trial clinico nella Sindrome di Marfan
Eloisa Arbustini, Università di Pavia - IRCCS Fondazione Policlinico San Matteo

Discussione con la platea

Filo Diretto con i Pazienti
Alessia Daturi, Fondazione Telethon

11.00 – 11.30 *Coffee break*

11.30 – 12.30 **SESSIONE POSTER 2: Incontro con i Ricercatori**

12.30 – 13.00 ***Fotografia dei partecipanti al Congresso***

13.00 – 14.00 *Pranzo*

14.30 *Partenza navetta per Verona aeroporto e stazione FS*

OPENING LECTURE

Talk 1

AN INTRODUCTION TO THE INTERNATIONAL RARE DISEASES RESEARCH CONSORTIUM (IRDIRC)

Irene Norstedt

Deputy Head of Unit Personalised Medicine, Health Research Directorate, DG Research and Innovation, European Commission, Brussels, Belgium
Irene.Norstedt@ec.europa.eu - http://ec.europa.eu/research/health/medical-research/rare-diseases/irdirc_en.html

The International Rare Diseases Research Consortium (IRDiRC) was launched in April 2011 and today it brings together some 30 public and private organisations from three continents.
IRDiRC teams up researchers and organisations investing in rare diseases research in order to achieve two main objectives, namely to deliver 200 new therapies for rare diseases and means to diagnose most rare diseases by the year 2020.
Maximising scarce resources and coordinating research efforts are key elements for success in the rare diseases field. Worldwide sharing of information, data and samples to boost research is currently hampered by the absence of an exhaustive rare disease classification, standard terms of reference and common ontologies, as well as harmonised regulatory requirements.
IRDiRC gathers organisations that share common goals and principles and have agreed to work in a coordinated and collaborative manner within a multinational consortium. A number of grand challenges will need to be addressed through collaborative actions to reach IRDiRC objectives and the 2020 goals:
- establish and provide access to harmonised data and samples;
- perform the molecular and clinical characterisation of rare diseases;
- boost translational, preclinical and clinical research.
This collaboration will also require harmonisation of policies related to research use, standardisation, and dissemination and a comprehensive policy agenda will be developed.

GENES AND MECHANISMS OF INTELLECTUAL DISABILITIES: PAVING THE WAY FOR POTENTIAL THERAPIES

Talk 2

INTELLECTUAL DISABILITY: LESSONS FROM 500 DIAGNOSTIC EXOMES

Han G. Brunner

Radboud University Medical Center Nijmegen, The Netherlands, h.brunner@gen.umcn.nl

The recent introduction of powerful new DNA sequencing machines has rapidly changed the study of inherited diseases. Interrogating all 22.000 human genes in one experiment, rather than one at a time, is now a practical possibility which we have explored in a first exploratory study on 500 patients with a number of different problems which often involve a genetic causes such as intellectual disability, movement disorders, blindness, and deafness. Our data suggest that it should now be possible to accurately molecularly diagnose a large fraction of patients with these conditions.
This has already changed the practice of medical genetics. Previously, the molecular test would have been the final step in the diagnostic journey. Molecular testing would be ordered, after an accurate clinical diagnosis had been obtained, supported by other tests, some of which might be invasive, expensive and cumbersome to the patient. Clearly, all this will now change, since it would be defensible to order an “exome” screen once a genetic condition is suspected, in order to provide the medical geneticist with a molecular differential diagnosis. From this, molecular list, further clinical investigation may then follow in order to confirm or refute suspected diagnoses.
It is clear, that any success of applying exome sequencing should not be limited to medical genetics.
An estimated 1 in 16 individuals has a rare disease or will develop a rare disease in their lifetime. Rare diseases are often not recognized and diagnosed, and long delays, misdiagnosis, and missed diagnosis occur frequently. About half of all patients with a rare disease visit multiple doctors over an extended period before they reach a specialist who recognizes their particular rare condition.
Given that a considerable proportion of rare diseases are based in genetics, one may envisage a scenario whereby patients with complex clinical presentation be included in a program for undiagnosed diseases where the next step would be exome sequencing. I propose that such a strategy would provide many accurate diagnoses, thereby reducing doctor’s delay, unnecessary invasive, costly and burdensome procedures, and allowing a prognosis and care pathway to be charted.
Since collectively, rare disease are not rare, exome sequencing will soon move to the front end of the diagnostic process for an increasing number of clinically complex conditions, amongst which are intellectual disability, neurodegenerative diseases, immune deficiencies and several others.

Talk 3

THE X-LINKED INTELLECTUAL DISABILITY PROTEIN TSPAN7 REGULATES EXCITATORY SYNAPSE DEVELOPMENT AND AMPAR TRAFFICKING

Maria Passafaro

CNR Neuroscience Institute, Milan, Italy, and DTI Dulbecco Telethon Institute

Defective formation or function of synapses in the CNS during development results in disorders of learning and memory, including autism and intellectual disability. X-linked intellectual disability (XLID) is a heterogeneous condition that is caused by single gene mutations on the X chromosome. Over half of the genes that are found to be mutated in XLID encode synaptic proteins involved in actin cytoskeleton rearrangement, synaptic plasticity, synapse formation or neurotransmission, although the precise roles of most of these genes remain unknown.
One of the genes responsible for XLID is TM4SF2 which encodes TSPAN7-member of tetraspanins family. Tetraspanins are evolutionary conserved membrane proteins that tend to associate laterally with one another and to cluster dynamically with numerous partner proteins in membrane microdomains (Tetraspanin-Enriched Microdomains, TEMs) but their function in brain is not clear. Here we describe that TSPAN7 promotes filopodia and dendritic spine formation in cultured hippocampal neurons, and is required for spine stability and normal synaptic transmission. We also identify PICK1 (protein interacting with C kinase 1) as a TSPAN7 partner. Remarkably, TSPAN7 regulates the association of PICK1 with AMPARs, and controls AMPAR trafficking.

Talk 4

FMR1 EXPRESSION AND FMRP FUNCTION AS POSSIBLE TARGETS OF PHARMACOTHERAPY IN THE FRAGILE X SYNDROME

Giovanni Neri, Elisabetta Tabolacci, Stella Lanni, Martina Goracci, Giorgia Mancano, Pietro Chiurazzi

Istituto di Genetica Medica, Università Cattolica del S. Cuore, Roma, Italy

The fragile X syndrome (FXS) is the leading cause of inherited intellectual disability, with a prevalence of approximately 1 in 3000 males. It is caused by an unstable mutation of the *FMR1* gene, consisting of expansion beyond 200 repeats of a CGG sequence in the 5′ UTR of the gene. Expansion is followed by methylation of the cytosines, including those of the promoter upstream, suppressing gene transcription. Absence of the protein product FMRP causes an excessive response of dendritic spine synapses to stimulation of metabotropic glutamate receptors, resulting in increased long term depression (LTD), as well as increased number of immature dendritic spines. Genetic homogeneity, apparent lack of structural damages to the nervous system, apparent reversibility of FXS manifestations in mouse model systems, support the view that FXS is amenable to treatment through drug-induced correction of the *FMR1* insufficiency. We have explored two possible approaches.
The first approach aims at reactivating the *FMR1* mutant gene by removing the epigenetic block that suppresses transcription. By treating FXS lymphoblastoid cells in vitro with the demethylating drug 5-azadeoxycytidine, we induced restoration of *FMR1* transcription and translation. The treatment induced not only DNA demethylation, but also methylation of lysine 4 and partial demethylation of

lysine 9 of histone H3, as well as acetylation of histones H3 and H4 in the *FMR1* promoter region. This euchromatic configuration is also seen in naturally occurring unmethylated full mutations that are found in rare individuals of normal intelligence. A modest reactivation of the mutant *FMR1* gene can also be induced by treatment with valproic acid. A preliminary clinical trial of this drug on 10 FXS individuals resulted in significant amelioration of their hyperactivity and attention deficit, as measured by the Conners scale (reviewed by Pirozzi et al. Am J Med Genet (part A) 155:1803–1816, 2011).
The second approach aims at compensating the absence of FMRP within the post-synaptic compartment of dendritic spines. Here, activation of metabotropic glutamate receptors (mGluR5) induces protein synthesis, which is normally downregulated by FMRP. Lack of FMRP results in excessive dendritic protein synthesis and increased LTD. This dysregulation can be corrected by mGluR5 inhibitors, such as AFQ056. This molecule was used in a double-blind, placebo-controlled, crossover study of 30 FXS adult subjects that demonstrated its efficacy in reducing hyperactivity and attention deficit. Interestingly, not all tested individuals appeared to respond to this treatment, but only those who were found, retrospectively, to be carriers of a completely methylated *FMR1* mutation (Jacquemont et al. Sci Transl Med 3: 64ra1, 2011).
Taken together, these results demonstrate that the concept of translational medicine can be successfully applied to the fragile X syndrome with a realistic expectation for the development of an effective pharmacotherapy within the foreseeable future.
Disclosure: Studies entailing the use of AFQ056 were done in collaboration with and supported by Novartis Pharma, AG.

Talk 5

MOLECULAR BASES AND IN VITRO MODELING OF CDKL5 DEPENDENT INFANTILE NEUROLOGICAL DISORDERS

Ricciardi Sara (1), Ungaro Federica (1), Hambrock Melanie (2), Rademacher Nils (2), Dario Brambilla (3), Sessa Alessandro (1), Charlotte Kilstrup-Nielsen (4), Carlo Sala (5), Kalscheuer Vera M. (2), Broccoli Vania (1)

(1) Istituto Scientifico San Raffaele, Milan, Italy, broccoli.vania@hsr.it
(2) Max Planck Institute for Molecular Genetics, Department of Human Molecular Genetics, Berlin, Germany
(3) Department of Human Physiology, University of Milan Medical School, Milan, Italy
(4) Laboratory of Genetic and Epigenetic Control of Gene Expression, Department of Structural and Functional Biology, University of Insubria, Busto Arsizio, VA, Italy
(5) Consiglio Nazionale delle Ricerche Neuroscience Institute, Milan, Italy

Mutations of the cyclin-dependent kinase-like 5 (CDKL5) and netrin-G1 (NTNG1) genes cause a severe neurodevelopmental disorder with clinical features that are closely related to Rett syndrome, including intellectual disability, early-onset intractable epilepsy and autism. We report here that CDKL5 is localized at excitatory synapses and contributes to correct dendritic spine structure and synapse activity. To exert this role, CDKL5 binds and phosphorylates the cell adhesion molecule NGL-1. This phosphorylation event ensures a stable association between NGL-1 and PSD95. Accordingly, phospho-mutant NGL-1 is unable to induce synaptic contacts whereas its phospho-mimetic form binds PSD95 more efficiently and partially rescues the CDKL5-specific spine defects. Interestingly, similarly to rodent neurons, iPSC-derived neurons from patients with CDKL5 mutations exhibit aberrant dendritic spines, thus suggesting a common function of CDKL5 in mice and humans.

THERAPEUTIC STRATEGIES FOR MENDELIAN DISORDERS

Talk 6

PHARMACOLOGICAL STRATEGIES TO RESCUE CHLORIDE TRANSPORT IN CYSTIC FIBROSIS

Luis J.V. Galietta

Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, Genova

Cystic fibrosis (CF), one of the most frequent genetic diseases in caucasian populations, is caused by reduced chloride and bicarbonate transport in the epithelial cells of various organs. In the lungs, the basic defect causes chronic bacterial infections and plugging of the airways by highly dense mucus secretions. Our aim is to develop novel pharmacological strategies to correct the basic defect in CF. To this purpose, we are considering two different targets: CFTR, the plasma membrane chloride channel protein that is altered by CF mutations, and the calcium-activated chloride channel (CaCC), which represents an alternative pathway to circumvent the basic defect. Our project has two specific aims: 1) the identification of drug-like small molecules to correct the molecular defect caused by F508del, the most frequent mutation affecting CF patients; 2) the development of strategies to upregulate TMEM16A protein, the major component of the epithelial CaCC.
The F508del mutation causes impaired maturation of the CFTR protein. Indeed, F508del-CFTR is detected as a misfolded protein during its biosynthesis and is rapidly degraded by the ubiquitin-proteasome system. Although a small fraction of the mutant protein reaches the plasma membrane, it is rapidly internalized and degraded. To rescue F508del-CFTR, we have screened chemical libraries with a high-throughput functional assay based on the halide-sensitive yellow fluorescent protein (HS-YFP). This approach has detected several active compounds that are able to improve F508del-CFTR targeting to the plasma membrane. In particular, we have identified small molecules, belonging to the class of aminoarylthiazoles, that cause a dual effect on F508del-CFTR. They improve protein trafficking but also enhance the intrinsic ion channel activity. In parallel, we are using the HS-YFP assay to screen a genome-wide siRNA library. The purpose is to identify genes whose silencing leads to rescue of F508del-CFTR. The screening has identified a series of proteins, involved in ubiquitination and sumoylation. Such proteins may represent novel drug targets in CF.
We are also studying the TMEM16A protein since it is a major component of epithelial CaCC. Interestingly, we have found that TMEM16A expression in the airway epithelium is particularly associated with mucus cell metaplasia, a condition of mucus hypersecretion that is typical of CF, asthma, and other chronic respiratory diseases. In particular, TMEM16A is highly expressed in goblet cells whereas CFTR is expressed in ciliated cells. Such results suggest that TMEM16A may be particularly required for mucin secretion. The separate localization of TMEM16A and CFTR requires further investigation to assess whether the former protein is a suitable drug target for CF therapy.

Talk 7

EXPLOITING ARTIFICIAL NUCLEASES FOR TARGETED TRANSGENE INTEGRATION IN HUMAN HEMATOPOIETIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS DERIVED FROM NORMAL DONORS AND SCID-X1 PATIENTS

Angelo Lombardo (1), Pietro Genovese (1,2), Claudia Firrito (1), Tiziano Di Tomaso (1), Giulia Escobar (1), Giulia Schiroli (1), Andrew R. Gennery (3), Lucia Sergi Sergi (1), Philip D. Gregory (4), Michael C. Holmes (4), Luigi Naldini (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy, San Raffaele Institute, Milan, Italy
(2) Vita-Salute San Raffaele University, Milan, Italy
(3) Newcastle Upon Tyne Hospital, Newcastle Upon Tyne, United Kingdom
(4) Sangamo BioSciences Inc., Richmond, CA, United States

Gene targeting by homologous recombination holds great promise for gene therapy as it may overcome the risks of insertional mutagenesis and uncontrolled transgene expression associated with the use of conventional gene transfer vectors. The development of artificial nucleases, such as Zinc Finger Nucleases (ZFNs), has brought the possibility of targeted integration and gene correction within the reach of gene therapy. A ZFN-induced DNA double strand break at a predetermined site of the genome can trigger homology-directed repair (HDR), a pathway that can be exploited to insert new sequences with high efficiency and specificity into the ZFN target site (Lombardo et al., Nat. Biotech. 2007; Gabriel*, Lombardo* et al. Nat. Biotech. 2001, *: equal contribution). Here we have exploited the ZFN technology to induce HDR-driven transgene insertion into a predetermined genomic site and used this strategy to insert transgene expression cassettes and correct mutations in Hematopoietic Stem/Progenitor Cells (HSPC) and induced Pluripotent Stem Cells (iPSC) from normal donors and X-linked Severe Combined Immunodeficiency (SCID-X1) patients, respectively. This disease, which is caused by mutations in the Interleukin-2 Receptor Common Gamma Chain (IL2RG) gene, is an ideal candidate to test the effica-

cy and safety of this novel approach, as HSPC-based gene therapy trials performed with randomly integrating vectors showed clinical benefits but also a high rate of leukemia due to insertional mutagenesis and uncontrolled transgene expression. To achieve ZFN-mediated targeted insertion in HSPC, we developed a combined gene delivery protocol based on integrase-defective lentiviral vectors to deliver a donor template DNA for HDR and mRNA nucleofection to drive a short but robust spike of ZFNs expression. By using this protocol we can either target a transgene expression cassette into the putative "safe harbor" AAVS1 locus (Lombardo et al., Nat. Methods 2011) or insert a functional corrective IL2RG cDNA downstream of its own endogenous promoter (Lombardo et al., Nat. Biotech. 2007) with unprecedented efficiency and high specificity in HSPC. Gene edited HSPC generated both erythroid and myeloid colonies in vitro and, upon transplantation into NSG mice, reproducibly gave rise to both myeloid and lymphoid lineages. Importantly, gene-targeted cells were also found in the mice within the primitive human hematopoietic compartment, thus demonstrating editing of the long-term repopulating stem cell. In parallel, we have explored the use of patient-derived iPSC as a potentially unlimited source of gene-corrected HSPC. We have established a strategy that allows correction of the IL2RG gene and safe reprogramming of these cells. Using ZFN technology we inserted a corrective IL2RG cDNA downstream of its own endogenous promoter in fibroblasts from SCID-X1 patients with high efficiency. Since fibroblasts do not express IL2RG, to identify the gene-corrected cells we included downstream of the corrective cDNA a loxP-flanked drug-selection cassette, and efficiently reprogrammed the selected cells to iPSC by using a single-copy, Cre-excisable Lentiviral Vector (LV) expressing the reprogramming factors (Camnasio et al., Neurobiol Dis. 2012). Transient Cre delivery resulted in near complete excision of the reprogramming LV and the selector cassette from the iPSC genome, demonstrating the feasibility of generating gene-corrected and reprogramming factor-free iPSC from SCID-X1 patients. Overall, these studies provide proof-of-principle for ZFN-mediated gene targeting and correction in wild-type and patient-derived HSPC and iPSC, and provide a path to the development of a more precise and safe gene therapy strategy for SCID-X1 and, conceivably, several other diseases.

Talk 8

HIGH CONTENT SCREENING FACILITY AT TIGEM: PHARMACOLOGICAL APPROACHES TO TREAT RARE GENETIC DISEASES

Diego L. Medina

Telethon Institute for Gene Therapy (TIGEM) – Naples, Italy

Lysosomes are ubiquitous intracellular organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways. The lysosome is involved in numerous diseases including lysosomal storage disorders (LSDs), and neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD). All these diseases share as a common feature the progressive accumulation of undigested macromolecules within the cell. Such accumulation ultimately results in cellular dysfunction that leads to clinical manifestations with variable association of visceral, skeletal, hematologic and, most importantly, severe neurological involvement. Whereas the unique solution for monogenic rare disorders is the replacement of the functional gene, the increasing discovery of the intracellular pathways involved in the pathogenesis of these diseases envisage new therapeutic alternatives to treat these devastating diseases, including pharmacological approaches.
High content screening (HCS) is a sophisticated method for drug discovery that combines all the molecular tools of modern cell biology with automated high-resolution microscopy and robotic handling. The technology allows the development of complex cell-based assays to measure multiple cell processes that simultaneously identify a relevant and specific cell phenotype, and to assess how this phenotype responds to chemical or genetic perturbations. The multiparametric analysis of these cell responses allows the simultaneous analysis of the effects of a given perturbant (small molecule or modifying gene) on a variety of molecular and cellular targets, including sub-cellular localization and redistribution of proteins. In contrast to biochemical screening, HCS detects the responses within the context of the intercellular structural and functional networks of normal and diseased cells, respectively.
Here we present the new HCS Facility at TIGEM that takes the advantages of both state-of-the-art HC technology and the knowledge we have gained on the cellular and molecular mechanisms of the pathogenesis of LSDs using cultured cells and animal models. We aim to, a) the developing of cell-based assays for the phenotyping of rare genetic diseases and, b) the discovery of new therapeutic approaches to treat neglected diseases, such as lysosomal storage disorders (LSDs), and other common neurodegenerative diseases.

Talk 9

A COMPUTATIONAL STRATEGY TO DISCOVER PHARMACOLOGICAL CHAPERONES WITH THERAPEUTIC POTENTIAL FOR RETINITIS PIGMENTOSA

Angelo Felline (1,2), Petra Behnen (2), Francesco Raimondi (1,2), Valeria Marigo (2), Francesca Fanelli (1,2)

(1) Dulbecco Telethon Institute (DTI)
(2) Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

A majority of the over 16,000 missense mutations linked to human diseases affect folding or trafficking, rather than protein function [1]. Many disease-linked mutations occur in integral membrane proteins, a class of proteins whose folding we know very little about. Conformationally based failure of a protein to achieve its functional structural state and normal cellular location as well as activation of Endoplasmic Reticulum (ER) stress are common contributors to the etiology and pathology of heritable human diseases. Defects in protein folding or trafficking are at the basis of the pathogenicity of rhodopsin mutations linked to Autosomal Dominant Retinitis Pigmentosa (ADRP). RP comprises a group of hereditary human diseases that are characterized by progressive retinal degeneration and severe visual impairment in as many as 1.5 million patients worldwide. Thus, ADRP-linked rhodopsin mutants fit in the protein-misfolding disease model suitable for treatments with pharmacological chaperones, i.e. ligands able to rescue proper protein fold [2]. Yet, the structural features of such mutants are obscure, which hampers rational drug design.
Rhodopsin, a member of the G protein Coupled Receptor (GPCR) superfamily of seven-transmembrane proteins, is the visual pigment molecule of rod cells that captures light and activates an electrical signal transmitted to the brain for vision [3]. Photon absorption by rhodopsin causes the cis-trans isomerization of the covalently bound retinal chromophore and the consequent formation of the signaling active state.
A structural model of rhodopsin misfolding was built by thermal unfolding simulations of 46 ADRP mutants combined with the graph-based Protein Structure Network (PSN) analysis implemented in the Wordom software [4], in line with previous mechanical unfolding simulations [5]. ADRP rhodopsin mutations share more or less marked abilities to affect the connectivity of selected amino acid residues, which act as hubs in the native structure network. These highly connected nodes are essentially located in the retinal binding site that participates in the stability core of the protein. In silico analysis was associated with an in vitro study of subcellular localization of different mutants expressed in Cos-7 cells.
The combination of structural and in vitro characterizations allowed us to select a number of representative rhodopsin mutants as targets of virtual screening. The latter considered over two-million compounds taken from the ZINC Database (http://zinc.docking.org/). A number of hit compounds were selected on the basis of docking score and ability to mimic the binding mode of retinal. In vitro testing of such compounds is underway.
The investigation provided insights into the structural fundamentals of the disease and is expected to discover pharmacological chaperones with therapeutic potential.
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KEYNOTE LECTURE

Talk 10

TIGEM GOES TRANSLATIONAL

Andrea Ballabio

Telethon Institute for Gene Therapy (TIGEM) – Naples, Italy

The main goal of the Telethon Institute of Genetics and Medicine (TIGEM) is to elucidate the molecular mechanisms underlying genetic diseases and to develop preventive and therapeutic strategies. Research at TIGEM is focused on three strategic programs, Cell Biology of Genetic Diseases, Systems Biology and Molecular Therapy. The Institute counts with 13 independent research groups and 7 research facilities tailored to support the Institute's research needs. Recently, TIGEM's research interest has reached a turning point with the immediate need to move from scientific discovery to clinical applications. Towards this goal, partnerships between non-profit organizations, such as Telethon, and pharmaceutical companies are of pivotal importance due to the complexity of expertize required and the high costs of translational studies and clinical trials. As of October 2012 the Telethon Foundation and Shire Pharmaceuticals have announced a 5-year partnership to support translational research at TIGEM. This partnership is based on TIGEM scientific discoveries in the fields of lysosomal function and cellular clearance, of the mechanisms underlying lysosomal storage disorders, neurodegenerative diseases and disorders of intracellular trafficking and on TIGEM development of novel gene therapy and cell-based drug screening approaches. Shire will invest approximately US$ 22 million in 5 years to support TIGEM research projects in these fields. The complementarity of TIGEM and Shire expertize and efforts will pave the way to the development of therapies for people suffering from several devastating diseases still lacking a cure.

Talk 11

IMAGINING NEW THERAPIES FOR PATIENTS WITH RARE DISEASES: SHIRE HGT AND TIGEM TRANSLATE THE FUTURE

Arthur Tzianabos

Shire Human Genetic Therapies, Shire Way, Lexington, MA, USA

Shire Human Genetic Therapies (Shire HGT) is a fully integrated biopharmaceutical company dedicated to helping patients with rare diseases lead better lives. The company has a long history of developing novel therapies for rare genetic diseases first as Transkaryotic Therapies and now as Shire HGT. Through its internal Research and Development capabilities, Shire HGT has developed enzyme replacement therapies (ERTs) for Fabry disease, Hunter Syndrome and Gaucher disease. All three ERTs were developed using the company's propriety gene activation technology in a human cell line. The company has a fourth product for patients suffering from hereditary angioedema (HAE). This product is a peptide that inhibits the bradykinin receptor and reduces the painful swelling associated with attacks in HAE patients. Shire HGT is actively developing novel approaches to bringing therapies forward for rare diseases through its own R & D efforts and in collaboration with other biotech companies, academic institutions, non-profit institutes, and patient organizations. In October 2012, Shire HGT and the Telethon Foundation announced a 5-year partnership to support translational research at TIGEM. This partnership is based on the recognition of the outstanding scientific advances TIGEM has made in the fields of lysosomal function and cellular clearance, of the mechanisms underlying lysosomal storage disorders, neurodegenerative diseases and disorders of intracellular trafficking and development of novel gene therapy and cell-based drug screening approaches. Through the combination of TIGEM's scientific expertise in discovering and translating novel therapeutic concepts and Shire HGT's non-clinical, clinical and manufacturing capabilities, the goal of the alliance is to bring much needed therapies for rare diseases to patients around the world.

ADVANCES IN CLINICAL TRIALS

Talk 12

THE DEVELOPMENT OF GIVINOSTAT FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY: FROM THE ANIMAL TO THE CLINIC

Paolo Bettica (1), Eugenio Mercuri (2)

(1) Clinical Research & Development, Italfarmaco S.p.A., Cinisello Balsamo, Italy
(2) Neuropsichiatria infantile, Universita Cattolica Sacro Cuore, Roma, Italy

Inhibition of Histone Deacetylases (HDAC) has beneficial effects in mouse models of muscular dystrophies indicating that HDACs are a pharmacological target for Duchenne Muscular Dystrophy (DMD). Givinostat is a potent HDAC inhibitor which is being developed for the treatment of DMD. Givinostat was first tested in a validated preclinical model of DMD, the mdx mouse. Results showed a dose- and concentration-dependent beneficial effect of the compound on muscle histological parameters affected by the disease such as inflammation, necrosis, fibrosis and myofiber regeneration. The histological effects translated into an improvement in function (treadmill test). The results obtained in the preclinical experiments formed the basis for the development of the ongoing clinical study in DMD ambulant children. In particular, the clinical study main objective is to confirm that the histological changes observed in the mdx model are obtained also in children treated with Givinostat. Moreover, a pharmacometric analysis of the preclinical results allowed us to identify Givinostat exposures expected to be efficacious and thus define the doses to be used in the clinical study. Finally, the study will also incorporate the assessment of a number of endpoints such as functional scales (6 Minute Walk Test, North Star, Upper Limb Scale), Magnetic Resonance, biomarker. In the end the study will allow us to understand the effect of Givinostat both at tissue and functional level. This study can be used as a paradigm of translational research. More specifically, the study illustrates how the parallel development of pharmacological and animal studies on one side, and, on the other side clinical related activities, such as patients registries, definition of standards of care and outcome measures have paved the way to facilitate the different steps leading to a clinical trial.

Talk 13

PHASE I/II CLINICAL TRIAL OF HEMATOPOIETIC STEM CELL GENE THERAPY FOR THE TREATMENT OF METACHROMATIC LEUKODYSTROPHY

Montini Eugenio (1), Lorioli Laura (1,2,3,4), Cesani Martina (1), Fumagalli Francesca (2,5), Plati Tiziana (1), Martino Sabata (5), Baldoli Cristina (7), Calabria Andrea (1), Canale Sabrina (2), Benedicenti Fabrizio (1), Vallanti Giuliana (8), Biasco Luca (1), Leo Simone (9), Kabbara Nabil (10), Zanetti Gianluigi (9), Rizzo William B. (11), Metha Nalini AL. (12), Cicalese Maria PIa (2,3), Casiraghi Miriam (2), Boelens Jaap J (13), Del Carro Ubaldo (5), Dow David J (12), Schmidt Manfred (14), Di Serio Clelia (4), Stupka Elia (15), Von Kalle Christof (14), Bordignon Claudio (4,8), Ciceri Fabio (3,16), Rovelli Attilio (17), Roncarolo Maria Grazia (1,2,3,4), Aiuti Alessandro (1,2,3,18), Sessa Maria (2,5), Naldini Luigi (1,4), Biffi Alessandra (1,2,3)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
(2) HSR-TIGET Pediatric Clinical Research Unit, San Raffaele Scientific Institute, Milan, Italy
(3) Pediatric Immunohematology, San Raffaele Scientific Institute, Milan, Italy
(4) Vita Salute San Raffaele University, Milan, Italy
(5) Neurology Unit, Department of Neurology, San Raffaele Scientific Institute, Milan, Italy
(6) Neuroradiology Unit, Head and Neck Department, San Raffaele Scientific Institute, Milan, Italy
(7) Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy
(8) Molmed S.p.A., Milan, Italy
(9) Distributed Computing Group, Center for Advanced Studies, Research and Development in Sardinia (CRS4), Pula, Italy
(10) Pediatric Hematology Oncology Division, Rafic Hariri University Hospital, Beirut, Lebanon
(11) Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE, USA.
(12) Molecular and Cellular Technologies, GlaxoSmithKline, Stevenage, United Kingdom

(13) Pediatric Blood and Marrow Transplantation Program, UMC Utrecht, Netherland
(14) Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany
(15) Center for Translational Genomics and BioInformatics, San Raffaele Scientific Institute, Milan, Italy
(16) Hematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, Milan, Italy
(17) Bone Marrow Transplant Unit, MBBM Foundation, Pediatric Department, Milano-Bicocca University at San Gerardo Hospital, Monza, Italy
(18) University of Rome Tor Vergata, Italy

Metachromatic Leukodystrophy (MLD) is an autosomal recessive lysosomal storage disorder caused by Arylsulfatase A (ARSA) deficiency and leading to severe demyelination, neurodegeneration and premature death of the affected patients. Currently, no treatment can halt the progression of this devastating disease. According to preclinical data demonstrating the safety and efficacy of hematopoietic stem cell gene therapy in the animal model of the disease, and based on the experience we acquired on the natural clinical course of the disease and its instrumental and clinical monitoring, on March 2010 a clinical trial based on transplantation of autologous hematopoietic stem cells transduced with lentiviruses (LVs) encoding ARSA was approved by the Italian Regulatory Authorities. The clinical protocol foresees the enrollment of 6 late infantile (LI) and 2 early juvenile (EJ) patients, in pre- and, in the case of EJ patients, early-symptomatic stage, in order to provide them a reasonable expectation of clinical benefit. The study objectives are the evaluation of the safety of the treatment, related to the myeloablative conditioning regimen employed and to the use of LVs, and of its efficacy by measuring patients' motor abilities and demyelination occurring in the nervous system through the use of validated instrumental readouts.
Until now seven patients have been enrolled and treated. Six of them had a biochemical, molecular and familiar history compatible with a diagnosis of LI MLD and have been treated in a pre-symptomatic stage of their disease. Only one patient, with a disease onset compatible with the EJ form of the disease, was treated in an early symptomatic stage. Thus far, we can report a favorable outcome of the transplant procedure with a good bone marrow recovery and the short/medium-term safety of both the conditioning regimen and stem cell transduction with LVs in all the treated patients. Moreover, we report stable sustained ARSA gene replacement to nearly exhaustive levels in the reconstituted hematopoiesis of the patients, resulting in supra-normal ARSA activity throughout the hematopoietic lineages and its reconstitution in the cerebrospinal fluid, the latter thus far documented in the first three treated patients. These findings are associated with substantial therapeutic benefit. Indeed, at the follow-up of the first three late infantile treated patients performed after the expected symptoms' onset (as defined according to disease onset in the affected older siblings) the disease had not appeared/progressed; furthermore they are rather experiencing a continuous motor and cognitive development, at odds with the natural disease course and their sibling anamnesis, and have a normal quality of life. These data are extremely encouraging, even if only the long-term follow-up of all the treated patients will confirm this favorable preliminary indication.

Talk 14

CLINICAL TRIAL OF HEMATOPOIETIC STEM CELL GENE THERAPY FOR WISKOTT-ALDRICH SYNDROME

Scaramuzza Samantha (1), Biasco Luca (1), Ferrua Francesca (2), Cicalese Maria Pia (2), Giannelli Stefania (1), Castiello Maria Carmina (1,3), Evangelio Costanza (2), Assanelli Andrea (2), Casiraghi Miriam (2), Bosticardo Marita (1), Rizzardi Paolo (4), Finocchi Andrea (5), Metin Ayse (6), Banerjee Pinaki P (7), Orange Jordan S (7), Biffi Alessandra (1,2), Villa Anna (1,8), Ciceri Fabio (9), Roncarolo Maria Grazia (1,2,3), Naldini Luigi (1,3), Aiuti Alessandro (1,2,5)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy
(2) Pediatric Immuno-hematology and Bone Marrow Transplant, San Raffaele Scientific Institute, Milan, Italy
(3) Università Vita-Salute San Raffaele, Milan, Italy
(4) MolMed SpA, Milan, Italy
(5) University of Rome "Tor Vergata", Rome, Italy
(6) Ankara Dispaki Children's Hospital, Ankara, Turkey
(7) Texas Children's Hospital, Baylor College of Medicine, USA
(8) IRGB-CNR, Milan, Italy
(9) Hematology and Bone Marrow San Raffaele Scientific Institute, Milan, Italy

Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. In patients lacking a compatible hematopoietic stem/progenitor cell (HSPC) donor, mismatched allogeneic transplantation or gene therapy with gamma-retroviral vectors provided clinical benefit but resulted in transplant complications and genotoxicity of the vector, respectively. We adopted a new protocol of HSPC gene therapy based on a self-inactivating lentiviral vector (LV) expressing the WAS protein under its endogenous promoter combined with reduced intensity conditioning (anti-CD20, busulfan and fludarabin). Three patients were treated with autologous bone marrow (BM) CD34+ cells transduced with highly purified lentiviral vector. The first patient received also mobilized peripheral blood transduced CD34+ cells to reach an adequate cell dose. Transduction of clonogenic progenitors was highly efficient (94.3 ± 5.3%), with a mean VCN/genome in bulk CD34+ cells of 2.1 ± 0.6. We then evaluated the engraftment of transduced cells in bone marrow and peripheral blood (PB) lineages and patients' immune functions. A robust multilineage engraftment of gene corrected cells was observed in the PB and BM at 1-1.5 years after treatment. Molecular tests showed the presence of gene modified cells in BM clonogenic progenitors (25-50%), BM myeloid lineages (VCN range: 0.29-0.78), PB granulocyte (VCN: 0.56±0.15) and lymphocytes (VCN range: 1.05-2.29) at the latest follow-up. WASp expression was detected in platelets, monocytes and at higher levels in lymphoid cells, as assessed by flow cytometric analyses. Proliferative responses to anti-CD3, NK cell cytotoxic activity, immune synapsis formation and Treg suppressive function were normalized after gene therapy. All patients are currently clinically well, independent from platelet transfusions, free from eczema and severe infections. High-throughput sequencing of vector integration sites after transplant demonstrated a highly polyclonal haematopoiesis. Importantly, we were able to directly compare the LV and gamma-retroviral vector insertion profiles in cells from gene therapy treated patients with the same disease background. This analysis highlighted significant differences in the genomic distribution of vector insertions between the two trials and showed that the LV gene therapy protocol did not induce in vivo selection of integrations near cancer genes or aberrant clonal expansions. The current clinical follow (1.4-2.4 years) and the cumulative data derived from other safety monitoring tests are consistent with an improved safety of LV gene therapy. In conclusion, LV-based HSPC gene therapy provides a new treatment option for WAS and, conceivably, other genetic blood disorders.

CARDIOMYOPATHY - FAILURE AND SUCCESS

Talk 15

EMERGING THERAPIES FOR DMD CARDIOMYOPATHY

Daniel J. Garry

Lillehei Heart Institute, University of Minnesota, Minneapolis, MN, USA 55455

Duchenne Muscular Dystrophy (DMD) is a common and deadly disease caused by dystrophin gene mutations. Treatment advances have improved survival however, DMD-associated cardiomyopathy has emerged as a major cause of death. Relatively few clinical studies have focused on DMD cardiomyopathy treatment and therefore uniform therapies have not been defined and/or broadly applied to this patient population. High resolution imaging technologies now allow us to interrogate and monitor the natural progression of DMD cardiomyopathy and to evaluate the impact of pharmacological therapies. The goal of our studies is to examine the role of the sympathetic nervous system and the renin-angiotensin-aldosterone system in the progression of DMD associated cardiomyopathy. We will examine the endogenous regenerative capacity of the adult heart focusing on the Abcg2 expressing cardiac SP progenitor cell population and the use of emerging technologies using cell therapy or iPSC derived cardiomyocytes for early diagnosis and treatment strategies. As a complementary approach, we have defined a role for Etv2 in promoting vascularization of the diseased heart. We have defined a Vegf/Flk2/Creb/Etv2 transcriptional network that promotes the endothelial molecular program. Collectively, these studies highlight new strategies aimed at restoring cardiovascular architecture and function in chronic diseases such as DMD cardiomyopathy.

Talk 16

CHANNELOPATHIES: MOVING TOWARD GENE THERAPY

Silvia G. Priori

Fondazione Salvatore Maugeri, Università di Pavia, Italy and Leon Charney Division of Cardiology, Cardiovascular Genetics, New York University, NY

Families affected by inherited life-threatening diseases are asking for a curative approach able to revert molecular, structural, and functional abnormalities of these diseases. This urgency is justified by the incomplete protection afforded by pharmacological therapy and by the frequent complications in the pediatric population of use of the Implantable Cardioverter Defibrillator that may be needed to reduce the risk of death.
Our clinical group is providing care to a large number of families with inherited arrhythmias both in Italy (Pavia) and in USA (New York) and therefore we appreciate the importance to advance therapies for channelopathies and therefore we recently committed to initiate a line of research targeted to the evaluation of gene therapy in one of the most lethal form on inherited arrhythmias called Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT). CPVT is an inherited arrhythmogenic disease associated with cardiac arrest in children or young adults. We identified that the gene for the autosomal recessive form is the cardiac Ryanodine receptor, but the most severe form is the recessive CPVT, characterized by the absence of cardiac calsequestrin 2, a protein that plays a pivotal role for the regulation of cytoplasmic calcium homeostasis in cardiac myocytes. The diagnosis of CPVT is most commonly established in subjects manifesting stress- or emotion-induced ventricular tachycardia and syncopal spells however sudden cardiac death may also be the first clinical sign of the disease. Recently, we engineered and characterized a CASQ2-/- mouse model that closely mimics the clinical phenotype of CPVT patients. We demonstrated that administration at birth of the CASQ2 gene delivery by adeno-associated viral vector was able to restore normal levels of calsequestrin and of the sister proteins Triadin and Junctin. Similarly, viral gene therapy was able to abolish cardiac arrhythmias in all mice. Mice were followed over time and at 52 weeks still maintain 100% protection from adrenergically induced arrhythmias. We therefore decided to explore whether such a remarkable therapeutic response could be reproduced in a Knock in mouse model CASQ2R33Q/R33Q treated with the AAV-CASQ2 construct despite the presence of the mutant protein that could hamper the polymerization of WT calsequestrin. Follow up data at 6-9-12 months confirmed that also in the knock in mice the viral gene therapy is effective in abolishing structural abnormalities (fragmentation of couplons), depletion of triadin and junctin as well as development of malignant arrhythmias.
These data are extremely encouraging and support our interest in moving toward a clinical efficacy trial of gene therapy in CPVT patients with mutations in the CASQ2 gene.

Talk 17

MELUSIN GENE THERAPY: AN INNOVATIVE APPROACH TO PREVENT FAMILIAL CARDIOMYOPATHIES

Mara Brancaccio, Cristina Rubinetto, James Cimino, Nicoletta Vitale, Guido Tarone

Department of Molecular Biotechnology and Health Sciences, University of Torino, Italy

Melusin is a muscle-specific gene required to activate a compensatory cardiac hypertrophy program. Its forced expression in heart of transgenic mice efficiently protects from left ventricle dilation in response to chronic pressure overload (De Acetis et al 2005) and myocardial infarct. Moreover, melusin levels are strongly reduced in the heart of human patients with dilated cardiomyopathy.
Melusin belongs to the family of small HSP molecular chaperons, it binds to the well known heat shock protein HSP90 and it is co-regulated both with HSP70 and HSP90. Molecular chaperones recognize partially denatured proteins and help them to retain correct function. Since mutations often affect protein folding, it has been proposed that the molecular chaperones can also function in buffering deleterious genetic mutations. Indeed experimental evidences indicate that chaperone overexpression allows the retention of catalytic activity of artificially mutated enzymes. We have, thus, set up a gene therapy protocol to test if overexpression of the muscle-specific melusin chaperone can buffer genetic mutations causing familial cardiomyopathies.
Two mouse models of cardiomyopathy, closely mimicking the human pathology are used. The first model replicates the Emery Dreifuss point mutation H222P in Lmna gene. Starting from 3 months of age, mutant mice develop dilated cardiomyopathy and muscular dystrophy with clinical features similar to those of human patients with the same mutation. A second model carries the W4R mutation in the MLP gene, a genetic defect with 1% frequency in Caucasian populations. Mutant mice develop hypertrophic cardiomyopathy at 12 months of age and the heart failure phenotype is characterized by almost complete loss of contractile reserve under catecholamine induced stress. Melusin will be expressed in the heart of these mutant mice either before or after the onset of the pathology allowing to evaluate the efficacy of the treatment both in preventing or treating the pathology. Cardio tropic adeno associated virus vector (AAV9) will be used for in vivo targeted gene delivery. Preliminary data of ongoing experiments will be presented.

Talk 18

INHERITED CARDIOMYOPTHIES: FROM PHENOTYPE-BASED TO GENETIC-BASED NOSOLOGY

Eloisa Arbustini

Centre for Inherited Cardiovascular Diseases, IRCCS Foundation Policlinico San Matteo, Pavia, Italy

Cardiomyopathies (CMP) are myocardial disorders in which the heart muscle is structurally and functionally abnormal, in the absence of other diseases sufficient to cause the observed myocardial abnormality. Based on phenotype, the current classification recognizes 4 major groups of cardiomyopathies:
- Dilated Cardiomyopathy (DCM, 1:2500) which is the commonest indication to heart transplantation;
- Hypertrophic Cardiomyopathy (HCM, 1:500) which is the commonest inherited heart condition in Europe affecting 1 million individuals;
- Arrhythmogenic (ARVC, 1:5.000) which is a common cause of Sudden Death in athletes and is currently classified as classical right (ARVC), biventricular and left dominant;
- Restrictive Cardiomyopathy (RCM), a very rare condition (1:100.000) characterized by abnormal relaxation and abnormal left ventricular filling, dilation of both atria and absence of significant LV hypertrophy.
More than 70% CMPs show familial. Clinical screenings of large series of consecutive families have shown that most familial CMP are inherited as autosomal dominant traits, with a minority of recessive (2%), X-linked recessive (5%) and matrilineal (3%) forms. The 4 major CMP phenotypes share: genetic heterogeneity, age-dependent penetrance, and potential overlapping of phenotypes, with different members of same families showing either HCM and DCM, or DCM and ARVC, or HCM and RCM.
More than 80 disease genes have been identified to date and the increasing number of genotyped families is providing the basis for revisiting the nosology of each major group of CMPs, based on the genetic cause rather on the major phenotype. Typical examples in the setting of DCM are dilated cardio-laminopathies (8% of all DCM) associated with high risk of life threatening arrhythmias, dilated cardio-dystrophinopathies (7% of consecutive DCM males) presenting with minor or clinically silent muscle involvement and predominant heart involvement leading to end-stage heart failure; sarcomeric HCM (about 90% of all HCM) and non sarcomeric HCM (10%) (HCM phenocopies), which include a broad group of multisystem diseases, affecting heart, muscle, brain, and other organs/tissues and including a variety of syndromes that require gene-based, specific diagnosis for treatment and prognostic stratification; desmosome ARVC caused by defect of genes coding desmosome proteins and non-desmosome ARVC caused by defects of genes also causing DCM phenotypes or left ventricular non-compaction (LVNC) such as LMNA or TAZ or LDB3; RCM broadly grouping in troponinopathies and desminopathies, two major groups of RCM with disease-specific traits, risk and systemic involvement.
Current treatments, medical interventional and surgical therapy (heart transplantation, LV remodeling) are based on clinical signs and symptoms and not on the specific disease: accordingly, we treat arrhythmias or heart failure, independently on the cause. Guidelines have been generated on phenotypes and are now largely inadequate to interpret the novel genetically driven clinical needs. The most impressive example is represented by cardio-laminopathies and ICD implantation. In LMNA patients the risk of SD is largely independent

on the severity of the LV dysfunction, which is one of the major criteria indicating ICD implantation according to existing guidelines.
A number of clinical warning is demonstrating that CMP patients cannot longer be classified and managed simply on their phenotype: CMPs caused by defects of different genes call for disease-specific diagnostic work-up, treatment strategy and prognostic stratification. Tools are available: by Sanger sequencing of 40 disease genes in DCM patients/families we have demonstrated that cardiac and extra cardiac clinical traits/ markers, additional to the major CMP phenotype (DCM or HCM or ARVC or RCM), may support a sustainable, clinically-oriented genetic testing in a large number of families. By NGS screening of 50 genes in 77 probands we obtained more than 40.000 non-synonymous, splice, frameshift and in frame variants, the majority not segregating in families, and with 80% confirmation by Sanger. Biotechnology cannot drive clinics; vice versa, our clinical skill and respect of rules of clinical genetics are the guarantee of the best use of new data for patients and families.
The future scenario for treatment innovation in inherited CMP includes at least four major avenues: 1. Development of novel drugs/molecules for subgroups of diseases sharing identical genetic causes; 2. Exploring, in experimental setting, the potential benefits of novel molecules (such as biological drugs) in subgroups of cardiomyopathies, based on specific interference of these molecules with the pathogenetic mechanisms of disease (typical example: cardio-laminopathies); 3. Old drugs for novel uses, when "new" molecular bases of "old diseases" meet recent discoveries of unexpected properties of old drugs (for example the anti-TGFbeta effects of Angiotensin II receptor 1 inhibitors); 4. Start medical treatments in preclinical phases of the CMP, before non-specific mechanisms of disease progression contribute to make the phenotype clinically overt. Under the EU project INHERITANCE (FP7 Project N. 241924) we are implementing a spontaneous clinical trial in mutation carriers with preclinical DCM (INHERITANCE-PRECARDIA). While waiting for novel molecules we need to make the best use of existing molecules and strategies: this is a urgent clinical need and a genetically-based nosology of CMP may largely contribute to this goal.

DECODING AN ENGINEERING MARVEL: INSIGHTS FROM GENETIC RENAL DISEASES

Talk 19

ON THE EDGE OF GLORY? POLYCYSTIC KIDNEY DISEASE RESEARCH AS A MODEL FOR MOVING FROM BENCH TO BEDSIDE

Gregory Germino

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, Bethesda, MD; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited diseases of man, affecting ~1/1000. Cysts arise at all stages of life, and gradually expand to replace normal renal parenchyma. This process results in end stage kidney failure (ESKF) in approximately half by the 6th decade, accounting for approximately 5% of all cases of ESKF. Hepatic cysts affect >80% and on rare occasions can necessitate liver replacement. Hypertension affects most, and intracranial aneurysms, which cluster in families, are an uncommon but often fatal extrarenal manifestation.
Therapies at present are limited to managing the complications. We have, however, made great progress in understanding the pathobiology of this disease and the role of the normal PKD gene products in regulating tubular morphology. Mutations in either of two genes, PKD1 and PKD2, cause all forms of the disease. Mutations appear to compromise gene function, and much data implicate a molecular recessive model as responsible for initiating cyst growth. PKD1 and PKD2 encode components of a receptor-channel complex that likely has ciliary and non-ciliary functions. Using syntenic mouse models, we have demonstrated unsuspected, complex development-stage specific consequences of Pkd1 inactivation that are linked to metabolic pathways. These models also have been used to show that PKD genes are essential for proper form and function of multiple other organs. In parallel to the lab-based studies, the ADPKD community has developed better tools for measuring progression of disease.
Armed with knowledge of pathways likely dysregulated in cystic epithelia, drugs that target the same, and non-invasive methods of assessing cystic progression, the community is on the cusp of changing the course of this disease. There have been several recent high profile clinical trials aimed at delaying progression, and many more under consideration. The community is abuzz with anticipation, and much activity is now focused on testing potential interventions. This new-found optimism has prompted some to declare that we now know enough to move aggressively ahead with testing treatments and focus less on investigating the underlying biology.
In this presentation, I will review progress in the field, discuss the recent clinical trials and demonstrate why we must continue to pursue basic mechanistic studies, even as we embrace the optimism and hopefulness of our patients.

Talk 20

UROMODULIN AND CHRONIC DISEASES OF THE KIDNEY

Luca Rampoldi

DTI c/o Molecular Genetics of Renal Disorders Unit, Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy

Uromodulin is a protein exclusively produced in the kidney and secreted in the urine where it represents the most abundant protein under physiological conditions. Its biological function is still elusive, though it has been associated with protection against urinary tract infections and kidney stone formation, ion transport and kidney innate immunity (1).
Mutations in UMOD, the gene encoding uromodulin, lead to rare autosomal dominant diseases collectively referred to as uromodulin-associated kidney disease (UAKD). They are characterised by progressive tubulo-interstitial damage, impaired urinary concentrating ability, hyperuricaemia, renal cysts and progressive renal failure. Through studies in cell models and in a transgenic mouse model of UAKD, we demonstrated that uromodulin mutations lead to ER retention of mutant protein, a primary event in the disease pathogenesis that precedes all other features (2). Uromodulin ER retention was never accompanied by induction of the unfolded protein response or apoptosis. Rather, some key pro-inflammatory markers were already upregulated well before the presence of any histological or functional renal damage, suggesting that UMOD mutations exert a non cell-autonomous proteotoxic effect that is significantly contributed by the inflammatory/fibrotic response. These data clearly demonstrate a gain-of-toxic function of uromodulin mutations and provide insight into the disease pathophysiology.
The importance of uromodulin in kidney (dys)function has been further enhanced by recent genome-wide association studies that identified uromodulin as a risk factor for chronic kidney disease (CKD) and hypertension (3). Our studies show that common risk variants in the UMOD gene are causally linked to these complex traits through modulation of gene expression.
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Talk 21

CELL BIOLOGY AND PHARMACOLOGY OF LOWE SYNDROME

Mariella Vicinanza (1), Antonella Di Campli (2), Elena Polishchuk (1), Michele Santoro (1), Maria Giovanna De Leo (1), MariaPaz Marzolo (3), and Maria Antonietta De Matteis (1)

(1) Telethon Institute of Genetics and Medicine, Naples, Italy
(2) Institute of Protein Biochemistry, CNR, Naples, Italy
(3) Pontificia Universidad Católica de Chile, Santiago, Chile

Mutations in the PtdIns4,5P_2 5-phosphatase OCRL cause Lowe syndrome, which is characterised by congenital cataracts, central hypotonia, and renal proximal tubular dysfunction. Previous studies have shown that OCRL interacts with components of the endosomal machinery; however, its role in endocytosis, and thus the pathogenic mechanisms of Lowe syndrome, have remained elusive. To gain insight into OCRL function, into the role of its PtdIns4,5P_2 5-phosphatase activity in membrane trafficking, and into the pathogenetic mechanisms of Lowe syndrome, we have analysed the endocytic compartments in kidney proximal tubular cells obtained from Lowe syndrome patients. Similarly, in kidney cell lines, we have studied the functional and ultrastructural consequences of the knock-down of OCRL-1 on multiple endocytic pathways. Our data indicate that through its PtdIns4,5P_2 5-phosphatase activity, OCRL maintains the

identity, structure, and function of early endosomes and the efficiency of trafficking pathways that traverse this compartment. These include the recycling of megalin, the multiligand receptor that drives uptake and absorption of protein in proximal tubular cells, and that undergoes mistrafficking in cells devoid of functional OCRL. The trafficking defects induced by OCRL mutations/depletion are caused by ectopic accumulation of $PtdIns4,5P_2$ in early endosomes, which in turn induces an N-WASP-dependent increase in endosomal F-actin. Finally, we have exploited the endocytic defects of cells deprived of OCRL to set up assays for high-content screening of small-molecule libraries to identify correctors of the endocytic mistrafficking as potential lead compounds for drug development for the treatment of Lowe syndrome.

Talk 22

CLC-5, AN ENDOSOMAL CHLORIDE–PROTON EXCHANGER MUTATED IN DENT'S DISEASE: A BIOPHYSICAL PERSPECTIVE

Michael Pusch, Silvia De Stefano, Michele Fiore, Antonella Gradogna, Elena Jeworutzki, Ilaria Zanardi, Giovanni Zifarelli

Istituto di Biofisica, CNR, Genova, Italy

BACKGROUND: Four of the nine human CLC proteins are plasma membrane Cl- channels whereas the other five are coupled 2 Cl- / 1 H+ antiporters localized in endo-/lysosomes (1). Mutations in CLC-5 lead to Dent's disease, characterized by low molecular weight proteinura (LMWPU), hypercalciuria, kidney stones and eventual renal failure (2). CLC-5 is localized in apical endosomes in the brush border in the proximal tubule (PT). Here, it is important for endocytosis, explaining the LMWPU phenotype (3). The other symptoms are indirectly caused by a defective PT handling of hormones involved in calcium homeostasis (3). Despite these physiological insights, the actual role of CLC-5 in endocytosis is unclear. Previously, it was thought that CLC-5 is a Cl- channel helping endosomal acidification, providing counter ions balancing charge accumulation by the H+ pump. However, it now clear that CLC-5 is a Cl-/H+ antiporter (4). A specific Glu residue (E211, "gating Glu") is essential for the antiporter function and mutating it to Ala (E211A) converts CLC-5 into a Cl- channel (4). Knockin-mice carrying this uncoupling mutation develop Dent's disease symptoms, demonstrating that the antiporter function cannot be replaced by a Cl- channel (5).
OBJECTIVES: Our group is investigating several biophysical aspects of CLC-proteins involved in genetic diseases (CLC-5, CLC-K, CLC-2). In this talk, we will focus on one aspect of CLC-5 that we have recently studied in detail (6). The Cl- / H+ exchange involves two critical Glu residues: the gating Glu (E211) controls access of Cl- from the extracellular space and its cyclical (de)protonation is critical for Cl-/H+ coupling (7); the proton Glu (E268) is the intracellular entry point for protons (8). The E268A mutant eliminates steady-state transport (9) but exhibits transient currents upon positive voltage steps (10). Such transient currents reflect charge movements within the transporter and their nature may reveal information on the molecular details of transport coupling.
RESULTS: From the dependence of the transient currents of E68A on pH and [Cl-], we conclude that they represent the movement of an intrinsic gating charge followed by the voltage dependent binding of extracellular Cl- ions. In addition, we found that the gating Glu mutation E211D abolishes stationary transport but displays transient currents which are shifted by 150 mV compared to those of E268A, identifying E211 as a major component of the charge movement. We suggest that that the initial events in the transport cycle are a movement of the gating Glu from the "external site" (Sext) to the "central site" (Scen) with accompanying displacement of a Cl- ion from Scen to the inside, followed by binding of an extracellular Cl- ion into Sext. These biophysical insights increase our molecular understanding of CLC antiporters. We hope that such an improved understanding aids in the development of strategies for the treatment of the diseases in which these proteins are involved.
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TRIAL READINESS IN NEUROMUSCULAR DISEASES

Talk 23

THERAPEUTIC POTENTIAL OF AAV-MICRODYSTROPHIN VECTORS FOR GENE THERAPY OF DMD

Jeffrey Chamberlain, Jane Seto, Julian Ramos, Guy Odom

Department of Neurology, University of Washington, Seattle, WA, USA

Duchenne muscular dystrophy (DMD) is among the most common lethal genetic disorder of children and is caused by mutations in the dystrophin gene. We are developing methods to deliver therapeutic genes to muscles throughout the body to either replace the missing dystrophin gene or to help compensate for the lack of dystrophin. We show that shuttle vectors derived from adeno-associated virus type 6 (AAV6) are able to deliver genes systemically to adult mice when injected directly into the vasculature. AAV6 delivery results in highly efficient gene expression in skeletal and cardiac muscle that persists for the lifespan of the mouse. However, the AAV shuttles have a limited carrying capacity, and as a result we have also been developing truncated versions of the dystrophin gene that can be carried by AAV yet retain sufficient functional capacity to halt dystrophy. A single injection of an AAV6/micro-dystrophin vector into the vasculature of adult, dystrophic mice results in elimination of dystrophic histopathology for the lifespan of the mouse. Studies are also underway to scale the procedures for the dog model of DMD. These studies revealed a cellular immune response directed against the AAV capsid proteins, but one that could be blocked by short-term immune suppression, leading to micro-dystrophin expression for at least two years in injected limb muscles of the *cxmd* model for DMD. In comparing transduction of canine muscles using AAV6 vs AAV9, we found that AAV6 is comparable or better than AAV9 in skeletal and cardiac muscles. Nor have we found evidence for neutralization or inactivation of AAV6 vectors in a survey of more than 30 dogs. These results suggest that a combination of intravascular AAV delivery coupled with transient immune suppression could lead to an effective therapy for DMD.

Talk 24

FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY: A WALK ON THE DARK SIDE OF THE (EPI)GENOME

Davide Gabellini

Dulbecco Telethon Institute and Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan, Italy

Only about 1% of the genome encodes for the 20000 human proteins, which are similar in number and largely orthologous to those found in organisms of significant lower complexity. On the contrary, the proportion of non protein-coding DNA has increased with developmental complexity reaching 98.5% in humans. Interestingly, up to two thirds of the human genome is composed of non protein-coding repetitive sequences. Furthermore, a significant portion of the epigenetic modifications is present in these regions and DNA repeats are dynamically transcribed in different cells and developmental stages producing a vast pool of non protein-coding RNA (ncRNA) molecules. Thus, ncRNAs produced by DNA repeats may hold the key to understanding the regulatory complexity inherent in advanced biological networks.
Long ncRNAs (lncRNAs) represent the most numerous and functionally diverse class of RNA produced by mammalian cells. Despite the growing interest on lncRNAs, they still remain poorly explored in terms of biological relevance, cellular function, mechanism of action and involvement in disease. We have recently contributed to this field through the identification of the first activating lncRNA involved in a human genetic disease: facioscapulohumeral muscular dystrophy (FSHD).
FSHD is the third most prevalent myopathy and is characterized by progressive wasting of facial, upper arm, and shoulder girdle muscles. The classical form of FSHD (FSHD1, MIM 158900) is not caused by mutation in a protein-coding gene. Instead, the disease is associated with a reduced copy number of the D4Z4 macrosatellite repeat mapping to 4q35. Several FSHD clinical features, such as the variability in severity and rate of progression, the gender bias in

penetrance, the asymmetric muscle wasting, and the discordance of the disease in monozygotic twins, strongly suggest the involvement of epigenetic factors. Accordingly, a number of epigenetic alterations have been reported in FSHD patients. While these features of the disease have been established for over a decade, the molecular mechanism through which D4Z4 repeats regulate chromatin structure and gene expression at 4q35 has remained elusive. We recently identified DBE-T, a chromatin-associated lncRNA produced preferentially in FSHD patients. We showed that DBE-T regulates the chromatin structure of the FSHD locus and the expression of FSHD candidate protein-coding genes.
Here, we will discuss our recent results regarding the regulation of DBE-T expression and how this ncRNA activates the epigenetic cascade leading to FSHD.

Talk 25

FROM THE BENCH TO THE CLINIC: WHAT WE HAVE LEARNT FROM THE ITALIAN NATIONAL REGISTRY FOR FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

Rossella Tupler

Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy

The practice of medical genetics requires a clear and definite evaluation of the significance of mutations and/or variations of DNA sequences for diagnosis, in order to provide prognostic information, and genetic counseling and to support research and clinical trials. This is particularly important for a progressive disease with unpredictable onset and a high variability of clinical expression, such as Facioscapulohumeral muscular dystrophy (FSHD). To address this fundamental question we have generated the Italian National Registry for FSHD (INRF), which collects clinical and molecular data from over one thousand independent families.
FSHD, a common myopathy, is considered an autosomal dominant disease. FSHD mutation has not been found in any protein-coding gene. Instead FSHD has been genetically linked to reduced numbers (≤8) of D4Z4 repeats at 4q35 combined with 4A(159/161/168) PAS haplotype. However, our most recent studies demonstrate that 1.3% of healthy individuals carry this molecular signature and 19% of subjects affected by FSHD do not carry alleles with reduced D4Z4 repeats. Thus the presence of D4Z4 reduction at 4q is not sufficient per se to cause disease. To unravel this unexpected genetic complexity, genotype-phenotype correlation was studied in 176 families in which FSHD and D4Z4 alleles of reduced size are present. The associations between clinical severity and size of D4Z4 allele, degree of kinship, gender, age, and 4q haplotype were evaluated. Overall, 32.2% of relatives did not display any muscle functional impairment. This phenotype was influenced by the degree of relation with proband, because 47.1% of second- through third-degree relatives was unaffected, while only 27.5% of first-degree family members did not show motor impairment. Male relatives were more severely affected than females. No 4q haplotype was exclusively associated with the presence of disease. Remarkably in 16% of families the disease expression was reported only in one generation, supporting the idea that the disease develops because of the presence of additional genetic defect(s). Indeed by extending clinical and molecular studies of FSHD patients and families we found other factors/pathologic conditions that might influence and modulate the disease expression.
In summary, our study indicates that a profound rethinking of the genetic disease mechanism and modes of inheritance of FSHD are now required and that entirely new models and approaches are needed. Our data point at the possibility that in the heterozygous state a D4Z4 reduction might produce a subclinical sensitized condition that requires other epigenetic mechanisms or a contributing factor to cause overt myopathy. In some rare cases, that could be by becoming homozygous and doubling the dose of a dominant factor at 4q35. In others, it might be by the simultaneous heterozygosity for a different hereditary myopathy, as suggested by many reports in which the FSHD contractions are found in association with a second molecular defect.
We believe that by broadening the scope of investigations, including next-generation deep sequencing in particular in families with asymptomatic and clinically affected members carrying the same FSHD allele, may finally lead to a understanding of the molecular pathogenesis of this complex disease with crucial implication for clinical practice and research in FSHD.

Talk 26

TRIAL READINESS IN PERIPHERAL NEUROPATHIES: THE CHARCOT-MARIE-TOOTH DISEASE PATHWAY

Davide Pareyson
on behalf of the Italian CMT Network

Clinic of Central and Peripheral Degenerative Neuropathies Unit, IRCCS Foundation, C. Besta Neurological Institute, Milan, Italy

Advances in our understanding of genetics and pathophysiology of Charcot-Marie-Tooth neuropathies (CMT) are creating great expectations in the field. Promising therapeutic approaches include rehabilitation therapy, curcumin, progesteron antagonists, neuregulin-1 III pathway regulation, high-throughput drug screening, and histon deacetylase 6 (HDAC6) inhibitors.
Translation of positive results of potential therapies in cellular and animal models to clinical trials in the human disease is long and difficult and requires a coordinated multidisciplinary approach. The preparation of the ascorbic acid (AA) multicentre trial in Italy and UK (funded by Telethon and co-funded by AIFA in Italy) was based on a ENMC workshop and on validation of outcome measures in CMT. All the AA trials worldwide were negative, revealed difficulties in detecting intervention efficacy, owing to slow disease progression, but provided a lot of important information on how to proceed in clinical trial design. A fundamental point is that we need to develop responsive outcome measures. We showed the limits of the CMTNS and the potentiality of foot dorsiflexion myometry (the most sensitive-to-change measure of the Italian-UK trial). Clinical research led to development of an updated version of the CMTNS, of a novel paediatric scale (CMTPedS) based on clinimetric methods, and of tests of outcome measures used in other disorders (activity monitors, 6MWT). Exploration of surrogate paraclinical outcome measures includes studies on computerised gait analysis and quantitative MRI, and biomarkers. We measured mRNA PMP22 levels in skin biopsies from 46 patients recruited in the ascorbic acid trial at study entry and end of study: we found no change over two years and no correlation with disease severity. The skin biopsy approach allows testing other potential biomarkers. National and international registries for neuromuscular disorders are being developed and are important for favouring recruitment in novel trials: a CMT registry, linked to the international registry, is starting in Italy thanks to the Registry Association, Telethon and the ACMT-Rete patients' association. Telethon funded many of these studies and made it possible the contribution of Italian centres to the international effort in finding a therapy for CMT.

Talk 27

TRIAL READINESS IN PERIPHERAL NEUROPATHIES: DEVELOPING A UNIFYING TREATMENT STRATEGY

Carla Taveggia (1), Maria Laura Feltri (2), Stefano C. Previtali (1), Lawrence Wrabetz (2), and Alessandra Bolino (1,3)

(1) Division of Neuroscience and INSPE San Raffaele Scientific Institute, Milan, Italy
(2) Division of Genetics and Cell Biology San Raffaele Scientific Institute via Olgettina 58, 20132 Milan, Italy
(3) Dulbecco Telethon Institute

CMT neuropathies, the most frequent human hereditary neuropathies, are associated with mutations in more than 30 genes. Modes of transmission include: recessive, dominant, or X-linked, with recessive diseases being more severe and of earlier onset. The severe disability and motor impairment derive mainly from progressive axonal damage and loss, with inefficient regeneration. Many CMT neuropathies display altered levels of myelination. Therefore therapeutical strategies aiming at restoring normal myelin levels could produce an effective approach for their collective treatment. Furthermore, as myelin is neuroprotective, restoring its normal levels could facilitate the maintenance of a functional Schwann cell-axon unit and reduce axonal damage, which is the ultimate cause of morbidity.
In the peripheral nervous system (PNS), the key molecule regulating all the aspects linked to myelination is Neuregulin1 (NRG1) type III. Above a threshold level of expression of NRG1 type III, axons are myelinated and the amount of myelin is proportional to the levels of axonal NRG1 type III. All NRG1 proteins are proteolytically

cleaved in their extracellular region. In particular the β-secretase BACE1 activates NRG1 type III enhancing myelination, while we recently showed that the pharmacologically accessible α-secretase TACE inhibits myelination. Our studies suggest the existence of a post-transcriptional mechanism modulating NRG1 activity and establish secretases as potential therapeutic target to modulate myelination in the PNS.
To confirm our hypothesis and identify a unifying treatment strategy for CMT hereditary neuropathies disorders, we explored the mechanisms of action of NRG1 type III and TACE in the array of preclinical animal models of CMT neuropathies we have generated and characterized over the years. Further we investigated whether NRG1 type III cooperates with components of the extracellular matrix and of the cytoskeleton to regulate myelination.
Our results indicate that in vitro and in vivo modulation of NRG1 type III levels is effective in modulating myelination in hyper- and hypo-myelinating neuropathies. We also have evidences suggesting that the extracellular matrix and the cytoskeleton modulate and integrate signals from NRG1 to regulate myelination. Further, the use of TACE inhibitors or activators can effectively control myelination in vitro in a Schwann cell neuronal coculture system. Preclinical trials are ongoing to validate our results in preclinical animal models of hypo and hyper-myelinating neuropathies.
With these studies we provide treatment prospects for subgroups of rare hereditary neuropathies and proof of principle that unifying strategies can be identified for rare diseases that in aggregate are common.

BLOOD DISORDERS: FROM GENETICS TO GENETIC THERAPIES

Talk 28

MUTATIONS IN THE 5'UTR OF ANKRD26 RESULT IN A "NEW" FORM OF INHERITED THROMBOCYTOPENIA THAT PREDISPOSES TO LEUKEMIA AND IS CHARACTERIZED BY THE PRESENCE IN PLATELETS AND MEGAKARYOCYTES OF A "NEW" CELL STRUCTURE

Carlo L. Balduini (1), Silverio Perrotta (2), Anna Savoia (3), Marco Seri (4)

(1) Medicina Interna, Fondazione IRCCS Policlinico San Matteo-Università degli Studi di Pavia, Pavia, Italy
(2) Dipartimento di Pediatria, Seconda Università di Napoli, Napoli, Italy
(3) Genetica Medica, Dipartimento di Scienze Mediche e Chirurgiche, Università degli Studi di Bologna-Policlinico S.Orsola-Malpighi, Bologna, Italy
(4) Dipartimento di Scienze mediche, chirurgiche e della salute, Università di Trieste-IRCCS Burlo Garofolo, Trieste, Italy

Thrombocytopenia 2 (THC2) is an autosomal dominant form of thrombocytopenia that, until recently, had been described in only 2 families: one in Italy and the other in North America. Linkage studies mapped the genetic locus to a ~ 8 Mb interval on chromosome 10p11-12, and mutations in three different genes were reported for this locus. In 2003, an aminoacid E167D substitution in the protein encoded by the *MASTL* gene was considered causative of the disorder in the North American family, and a transient knockdown of *MASTL* in zebrafish resulted in reduction of circulating thrombocytes (1). Nevertheless, no other case of THC2 has been subsequently found to have mutations in the same gene. More recently, an *ACBD5* missense change was reported as possibly disease-causing in the original Italian family (2). Finally, in this last family, as well as in 8 additional unrelated pedigrees without alterations in *MASTL,* several mutations in a specific sequence of the *ANKRD26* 5'UTR (from c.-134 to c.-113) were described (3). After this report, 12 different non-coding nucleotide changes in the 5'UTR of *ANKRD26* were identified in 21 families out of a cohort of 210 thrombocytopenic pedigrees. Thus, *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT) is one of the most frequent forms of inherited thrombocytopenia (4). Current genetic data strongly argue in favor of THC2 as a disorder due to specific alterations of *ANKRD26*, and the hypothesis that THC2 is a genetically heterogeneous should be supported by testing the affected members of the *MASTL*-mutated North American family for 5'UTR *ANKRD26* mutations.
The mechanism by which *ANKRD26* mutations lead to the disease is unknown, although Luciferase reporter assays suggested that they may enhance expression of gene (3). This hypothesis is consistent with the observation that mice with partial inactivation of *Ankrd26* are not thrombocytopenic and that a heterozygous deletion of about 31.5 Kb involving the last five exons of the gene in the James D. Watson's genome is not associated with a thrombocytopenic phenotype (3).
Most of patients described so far had moderate thrombocytopenia (mean platelet count 47 x10^9/L) and mild bleeding tendency. In contrast to the majority of inherited thrombocytopenias, which are characterized by large platelets, mean platelet volume was normal or slightly reduced. Peripheral blood film examination, in vitro platelet aggregation and flow cytometry of platelet GPs did not show consistent defects. Bone marrow examination and serum thrombopoietin levels suggested that thrombocytopenia is derived from dysmegakaryopoiesis, since many small and dystrophic megakaryocytes, often with hypolobulated nuclei, were observed.
Interestingly, the prevalence of acute leukemia among patients with this disorder was more than 30 time higher than in normal population, raising the suspicion that mutations in the *ANKRD26* 5'UTR expose to the risk of hematological malignancies (4). This suspicion is increased still further by the identification of large amounts of Particulate Cytoplasmic Structures (PaCSs) in platelets and megakaryocytes of patients with *ANKRD26*-RT (5). These recently identified cell structures, consisting of polyubiquitinated proteins and proteasome, have been observed so far in a number of solid cancers, in preneoplastic gastric lesions and in the neutrophils of Shwachman-Diamond syndrome, a genetic disease with neutropenia and increased leukemia risk.
Based on the findings reported above, further study of *ANKRD26*-RT promises not only to improve knowledge of inherited thrombocytopenias, but also to clarify the functional significance of PaCSs and the role of *ANKRD26* in oncogenesis.

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Talk 29

HEPCIDIN AT THE CROSSROAD BETWEEN HEMOCHROMATOSIS AND GENETIC IRON DEFICIENCY

Clara Camaschella, Antonella Nai, Marco Rausa, Alessia Pagani, Laura Silvestri

Università Vita-Salute and IRCCS San Raffaele, Milan, Italy

Genetic studies of hemochromatosis have contributed to identify the liver peptide hormone hepcidin as the key regulator of systemic iron homeostasis. Binding to and internalizing ferroportin, the sole cellular iron exporter, hepcidin regulates iron release from enterocytes and macrophages to plasma. *In vitro* studies and mice models have identified the IL6-STAT3 signaling as the pathway that increases hepcidin transcription in inflammation and the BMP6-SMAD signaling as the iron responsive pathway. The latter feedback mechanism that limits excess iron is deficient in hemochromatosis and in iron loading anemias, as exemplified by beta-thalassemia. Mutations of hepcidin, the BMP coreceptor hemojuvelin, HFE and TFR2 cause hemochromatosis of different severity, correlated to the degree of inability to increase hepcidin. Hepcidin transcription is suppressed in all conditions that require iron, such as iron deficiency, hypoxia and erythropoiesis expansion. Hepcidin suppression is mediated by the "erythroid regulator", whose nature remains elusive. The serine protease matriptase-2 encoded by *TMPRSS6* is the only hepcidin inhibitor known *in vivo.* TMPRSS6 inactivation in humans and mice causes iron refractory iron deficient anemia (IRIDA), since hepcidin excess blocks intestinal absorption of dietary and pharmacological iron. Membrane hemojuvelin is a substrate of TMPRSS6 and hemojuvelin cleavage accounts for the downregulation of the BMP-SMAD pathway in iron deficiency (Silvestri et al, Cell Met 2008;8:502-11), a mechanism disrupted in IRIDA (Silvestri et al, Blood 2009,113:5605-8)
TMPRSS6 haploinsufficiency in mice predisposes to iron deficiency (Nai et al, Blood 2010,116:851-2). In humans GWAS have shown that *TMPRSS6* genetic variants are associated with erythrocytes traits and iron parameters. We observed that the minor allele of the common *TMPRSS6* rs855791, found associated with low MCV and MCH, induces a higher hepcidin transcription in an *in vitro* assay and associates with high hepcidin levels in a large study of normal individuals (Nai et al, Blood 2011;118:4459-621).
Since TMPRSS6 is the only hepcidin negative regulator known in mammals we reasoned that deletion of *TMPRSS6* would be of bene-

fit to both hemochromatosis and beta-thalassemia, the latter being characterized by low hepcidin despite iron overload, because of the dominant negative effect of the expanded erythropoiesis. Genetic loss of *Tmprss6,* increasing hepcidin expression, improves *TfR2*-hemochromatosis in mice and ameliorates the phenotype of Hbbth3/+, a murine model of thalassemia intermedia (Nai et al, Blood 2012,119:5021-9). Along these lines recent preclinical studies have shown the potential of RNAi targeting *Tmprss6* for treatment of low-hepcidin conditions.

Talk 30

RNA-BASED THERAPEUTIC APPROACHES FOR BLOOD COAGULATION FACTOR DEFICIENCIES CAUSED BY SPLICING MUTATIONS

Mirko Pinotti, Franco Pagani

Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy

Intervention at the pre-mRNA splicing level represents a promising therapeutic approach for genetic diseases as it permits restoration of gene expression while maintaining gene regulation in physiological tissues, and overcomes limitations related to vector-mediated delivery of large genes. Increasing attention has been given to the U1 small nuclear RNA (U1snRNA) that, in the earliest splicing step, mediates recognition of the donor splice site (5'ss) by the ribonucleoprotein U1snRNP. Studies in various cellular models of demonstrated that engineered U1snRNAs can rescue splicing impaired by 5'ss mutations.
We explored the modified U1snRNA in severe coagulation factor VII (FVII) and IX (FIX, hemophilia B) deficiencies, in which splicing mutations are relatively frequent (15%). FVII/FIX deficiencies may lead to life-threatening bleeding and their treatment still presents several drawbacks. The U1snRNA-mediated in these diseases approach can be particularly beneficial since even modest increase of functional protein levels would result in improvement of clinical phenotypes.
As first model we chose the FVII deficiency caused by the c.840+5G>A mutation in the F7 IVS7 5'ss. Studies in cellular models indicated that the modified U1snRNA+5a (U1+5a), which restores complementarity to the mutated 5'ss, efficiently rescued FVII splicing and pro-coagulant function (8-10% of FVIIwt). To assess the U1+5a efficacy *in vivo* we created a novel mouse model of human FVII (hFVII) deficiency by liver-restricted expression, either transient (by non-viral vectors) or prolonged (by adeno-associated viral [AAV] vectors), of the mutated splicing-competent hFVII minigene (FVII+5A) in mice. In both experimental systems, hFVII expression was detectable in mice only upon co- injection of vectors for the U1+5a. In particular, correct hFVII transcripts were detectable in hepatocytes. Transient U1+5a co-expression resulted in hepatocyte localization of hFVII and secreted levels up to 367 ng/ml (~17% of wt-hFVII). Prolonged and vector dose-dependent hFVII levels in plasma (from undetectable up to 30 ng/ml) was demonstrated via delivery of AAV-FVII+5A and AAV-U1+5a viral vectors.
At variance from FVII deficiency, Hemophilia B provided us with several *F9* splicing mutations potentially approachable with U1snRNAs. By minigene splicing assays we established the disease-causing mechanism of 25 mutations at 5'ss, which resulted in exon skipping or cryptic 5'ss activation. U1snRNAs with increased complementarity to mutated 5'ss efficiently corrected several 5'ss defects. Intriguingly, they also corrected some acceptor splice site (3'ss) mutations. Through splicing-competent cDNA constructs we also demonstrated restoration of secreted FIX levels with coagulant activity (from undetectable to to 100%).
To improve specificity for *F9* gene, we designed a panel of U1snRNAs targeting non-conserved intronic sequences downstream of the 5'ss (Exon Specific U1snRNA, ExSpeU1). We found a gradient of rescue efficacy, which decreased with the 5'ss distance. Intriguingly, the best ExSpeU1 (ExSpeU1-FIX+9) remarkably rescued FIX biosynthesis and function (~90%) impaired by different 5'ss or 3'ss mutations.
CONCLUSIONS: For the first time, we demonstrated that a unique ExSpeU1 can restore gene expression impaired by different splicing mutants, and the increase of FVII/FIX levels, if achieved in patients, would be far beyond the therapeutic threshold. Moreover, we provided the first *in vivo* proof-of-principle of the therapeutic potential of the U1snRNA-mediated correction of splicing mutations, a frequent cause of all human genetic diseases.

Talk 31

GENE THERAPY FOR NON-LETHAL DISORDERS: THE PARADIGM OF BETA-THALASSEMIA

Giuliana Ferrari

San Raffaele-Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute and "Vita-Salute" San Raffaele University Medical School, Milan, Italy

Beta-thalassemia (ß-thal) is a severe congenital anemia caused by reduced or absent β globin chain production of the adult hemoglobin tetramer ($\alpha_2\beta_2$, HbA). It represents the most common autosomal recessive syndrome to cause a major health problem worldwide. Its global estimated annual birth incidence is 40.000/year and it occurs at a high frequency in a line stretching from Mediterranean littoral to Middle East and Southern Asia countries. More than 200 mutations leading to the disease have been described, affecting all the steps related to the expression of the b-globin gene.
Profound anemia is the most frequent and early clinical manifestation of the severe form of ß-thal major. Consequently, children affected and not appropriately treated show the stigmata of chronic hemolysis, the profound local and systemic effects of a rapid expansion of erythroid bone marrow mass and the organ damage due to iron overload.
Treatment of ß-thal is essentially supportive. Patients require a life-long transfusion regimen combined with iron chelation therapy to reduce hemosiderosis that is ultimately fatal if not continuously treated. Before the introduction of regular transfusion and chelation therapy, ß-thal used to be a rapidly fatal disease. Nevertheless, in the last few decades, the life expectancy of thalassemic patients has progressively increased.
The widespread acceptance of chronic hypertransfusion in association with continuous iron chelation as the therapy of choice for severe ß-thal has altered the typical clinical course for patients in areas of the world with sufficient resources to support adequate healthcare, transforming a lethal disease of infancy into a chronic disease of adulthood. For these reasons, we have a dramatic difference in both survival and life expectancy of patients from regions with limited resources compared to those from wealthier countries.
At present, the only curative approach is represented by allogeneic hematopoietic stem cell transplantation (HSCT), which, however, is limited by HLA compatibility and toxicity due to graft versus host disease, graft rejection and immunosuppressive regimens required.
Medical management and HSCT are both therapeutic options that could improve the quality of life and survival of thalassemic patients. Nevertheless, they are both burden by complications and limitations, outlining the need for testing innovative curative approaches.
For these reasons ß-thal has long been studied as a candidate for treatment by gene therapy, i.e. the autologous transplantation of genetically-modified hematopoietic stem cells. The development of HIV-derived lentiviral vectors (LV) and the optimization of hematopoietic stem cells transduction have provided significant contributions to this field, leading to the application of LVs expressing the human ß-globin gene in preclinical models and to the treatment of the first patient in France.
In this presentation, the major contributions from HSR-TIGET to the field, from development and production of ß-globin vectors to disease correction in preclinical models, as well as relevant issues related to the future clinical translation, like the choice of the source of hematopoietic stem cells and the demonstration of safety, will be discussed.

Talk 32

GENETIC THERAPY FOR HEMOGLOBINOPATHIES: FROM THE BENCH TO THE BEDSIDE

Punam Malik

Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

Sickle cell disease (SCD) and β-thalassemias are common monogenic blood disorders with potentially devastating consequences. In SCD, the chronic episodic vascular occlusions cause cumulative damage to organs and tissues and have an enormous impact on patient's quality of life, on the healthcare system, and on the lifespan of individuals with SCD. In β-thalassemia, patients are dependent on chronic transfusions for a lifetime. The only current curative therapy is an allogeneic hematopoietic stem cell transplant (HCT)

from a matched sibling donor, but is restricted by the availability of matched related donors and has potential complications such as graft versus host disease, graft rejection, and late effects, such as sterility. Gene therapy of autologous hematopoietic stem cells followed by transplant could result in a one-time cure, avoid adverse immunological consequences and not be limited by availability of donors; it may also be feasible with a reduced-intensity chemotherapy conditioning regimen, thereby lowering toxicity. We show correction of SCD and β-thalassemia mouse models. Based upon our preliminary data, we propose that gene transfer of gamma-globin lentivirus vector into CD34+ cells of subjects with SCD and β-thalassemia followed by autologous transplant following chemotherapy will be safe, feasibility and show potential efficacy. The eventual goal of gene transfer is to offer a patient a one-time ex vivo correction of sickle or thalassemia hematopoietic stem cells and their autologous transplant and circumvent the immunological consequences such as graft rejection and graft versus host disease associated with allogeneic transplant. We are proposing a phase I/II pilot gene transfer trial that will study the safety, feasibility and efficacy of a) obtaining sufficient CD34+ cells via a bone marrow harvest in patients with SCD and via apheresis in patients with β-thalassemia; b) ex-vivo gene transfer of the γ-globin lentivirus vector into the sickle or thalassemia CD34+ cells followed by c) chemotherapy conditioning and d) engraftment and efficacy of autologous gene modified CD34+ cells, when transplanted back into subjects with SCD and β-thalassemia. Preclinical data on HSC correction in mouse and human models of SCD and thalassemia and the design of the two gene therapy trials will be presented.

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GIACCA MAURO

TELETHON RESEARCH SERVICES/CORE FACILITIES

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PESOLE GRAZIANO

TELETHON INSTITUTE OF GENETICS AND MEDICINE

ABSTRACT N. 1

TTIGEM - Neuromuscular Diseasess		
Principal Investigator	**PARENTI GIANCARLO**	
Telethon grant N.	**TGM11MT4**	
Total budget approved €	**192.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

SMALL MOLECULE-BASED THERAPIES FOR LYSOSOMAL STORAGE DISEASES

Parenti Giancarlo (1,2), Cardone Monica (1), Porto Caterina (1,2), Rossi Barbara (1), Donaudy Francesca (1), Tarallo Antonietta (1), Pignata Antonella (1)

(1) Telethon Institute of Genetics and Medicine - Via P. Castellino 111, 80131 Naples; Tel: 081 6132227; E-mail: parenti@tigem.it
(2) Department of Pediatrics, Federico II University, Naples

Enzyme replacement therapy (ERT) is currently considered the standard of care for the treatment of several lysosomal storage diseases (LSDs), but, despite success in some of these diseases, this approach has limitations and leaves important clinical issues unsolved. Approaches based on the use of small-molecule drugs may have advantages compared with ERT, such as a better bioavailability and oral administration. Pharmacological chaperone therapy (PCT) is based on the use of ligands that prevent misfolding and degradation of mutated proteins. Substrate reduction therapy (SRT) is aimed at reducing the flux of substrates to lysosomes through the inhibition of specific steps in their synthetic pathways. We have evaluated the potential of these approaches in three different disease models, Pompe disease (PD) due to alfa-glucosidase (GAA) deficiency, Fabry disease (FD) due to alfa-galactosidase A deficiency, and mucopolysaccharidosis IIIA (MPSIIIA) due to N-sulfoglucosamine sulfohydrolase deficiency.
Much of the work has been done on PCT in PD. We have shown that the combination of PCT and ERT in this disorder has a synergistic effect. These studies opened the way to a clinical trial on the combination of the chaperone NB-DNJ (Miglustat) and ERT with human recombinant GAA (rhGAA) in PD. Thirteen PD patients have been enrolled in this study. Preliminary results show that the combination of PCT and ERT results in increased GAA peak activities (measured in dried blood spots) in all patients, compared to the activities obtained in the same patients with ERT alone (see also Abstract No 84).
We have recently identified three novel allosteric chaperones for GAA. The strategy used for the identification and characterization of these compounds was based on the combination of biochemical studies and of a computational analysis of their interactions with GAA. The identification of these molecules is innovative as they are the first example of chaperones that do not interact with the catalytic site of the enzyme, and thus are not potential inhibitors of the enzyme.
We have demonstrated that the synergy between ERT and PCT is not only limited to PD, but is also observed in FD. These studies support the idea that combination protocols may be extended to the treatment of any LSDs for which ERT and chaperones are available. This will possibly translate into improved efficacy of ERT in LSDs.
While this synergy is well documented both in vitro and in vivo, the mechanisms underlying this effect are poorly understood. Studies currently in progress show that chaperones improve the stability of recombinant enzymes as a function of pH and increase their lysosomal targeting after their uptake by cells.
We are also exploring the potential of a different approach, SRT, for the treatment of MPSIIIA. These studies are focused on the use of a molecule able to reduce the synthesis of proteoglycans and glycosaminoglycans in MPSIIIA fibroblasts.

ABSTRACT N. 2

TIGEM - Other Genetic Diseases		
Principal Investigator	**BALLABIO ANDREA**	
Telethon grant N.	**TGM11CB6**	
Total budget approved €	**560.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

MODULATION OF CELLULAR CLEARANCE IN LYSOSOMAL STORAGE DISORDERS

Settembre Carmine (1,2,3), Annunziata Fabio (1), Calcagnì Alessia (1), D'Orsi Luca (1), De Cegli Rossella (1), Florio Francesca (1), Mansueto Gelsomina (1), Montefusco Sandro (1), Peluso Ivana (1), Prezioso Carolina (1), Saide Assunta (1), Medina Sanabria Diego Luis (1), Spampanato Carmine (1), Ballabio Andrea (1,2,3,4)

(1) Telethon Institute of Genetics and Medicine (TIGEM), Via P. Castellino 111, 80131 Naples, Italy
(2) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA
(3) Jan and Dan Duncan Neurological Research Institute, Texas Children Hospital, Houston, TX 77030, USA
(4) Medical Genetics, Department of Pediatrics, Federico II University, Naples 80131, Italy

The lysosome plays a major role in cellular clearance. As cellular catabolic needs may vary depending on tissue type, age and environmental conditions, we postulated the presence of a system allowing the coordination of lysosomal activity. Using a systems-biology approach we discovered a gene regulatory network (CLEAR: Coordinated Lysosomal Enhancement And Regulation) that controls lysosomal biogenesis and function and a master gene, the bHLH-leucine zipper transcription factor TFEB, which binds to CLEAR target sites in the promoter of lysosomal genes and positively regulates their expression. TFEB overexpression induces lysosomal biogenesis and promotes cellular clearance (Sardiello et al. Science 2009). We also demonstrated that TFEB is directly involved in the regulation of two important cellular processes that are mediated by the lysosome: autophagy (Settembre et al. Science 2011) and lysosomal exocytosis (Medina et al. Dev. Cell, 2011). TFEB overexpression promoted cellular clearance in several murine models of lysosomal storage diseases (LSDs), both in cell culture and in vivo (Medina et al. Dev. Cell, 2011). Recent studies in our laboratory revealed that TFEB co-localizes with the master growth regulator kinase mTOR on the lysosomal membrane. When nutrients are present, phosphorylation of TFEB by mTOR, which occurs on the lysosomal surface, inhibits TFEB activity. Conversely, pharmacological inhibition of mTOR, as well as starvation and lysosomal disruption, activate TFEB by promoting its translocation to the nucleus. These data identify an entirely novel, nutrient-sensitive, lysosome-to-nucleus signaling mechanism that senses and regulates the lysosome via mTOR and TFEB (Settembre et al. EMBO J, 2012). In conclusion, we identified a novel, and potentially druggable, mechanism that exerts a global control on cellular clearance and on cellular energy metabolism.

ABSTRACT N. 3

TIGEM - Other Genetic Diseases		
Principal Investigator	**FRALDI ALESSANDRO**	
Telethon grant N.	**TGM11MT5**	
Total budget approved €	**90.000**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2011**

MODIFYING LYSOSOMAL ENZYMES TO IMPROVE SECRETION AND BRAIN DELIVERY

Sorrentino Nicolina Cristina (1), D'Orsi Luca (1), De Leonibus Elvira (1), Ballabio Andrea (1,2), Fraldi Alessandro (1)

(1) Telethon Institute of Genetics and Medicine, Via P. Castellino 111, 80131 Naples, Italy, Tel. +39 081 6132462, Fax +39 081 5609877, E-mail: fraldi@tigem.it
(2) Department of Pediatrics, Federico II University, Via S. Pansini 5, 80131 Naples, Italy

Mucopolysaccharidoses (MPS) type IIIA is a lysosomal storage disorder (LSD) caused by mutations in the sulfamidase gene. The brain represents the principal target of pathological lesions in this disorder but no effective therapies exist for the treatment of brain pathology.
We generated an engineered sulfamidase capable to be highly secreted and to cross the blood brain barrier (BBB). The engineered sulfamidase contains the low-density lipoprotein receptor (LDLR)-binding domain of apoliproprotein B (ApoB-BD), which confers to the modified enzyme the capability to cross the BBB by LDLR-mediated transcytosis. The chimeric sulfamidase also contains an alternative signal peptide (sp) belonging to the iduronate sulfatase (IDS), a highly secreted protein, which allows the modified enzyme to be efficiently secreted from the liver. AAV serotype 8 vectors were used as vehicle for the systemic delivery and liver targeting of the chimeric sulfamidase (hIDSsp-SGSHflag-ApoB-BD). We demonstrated that the intravenous injection of these vectors in adult MPS-IIIA mice converted the liver into a factory organ for the sustained

release of the modified sulfamidase in the blood stream of injected animals. Moreover, we detected a significant increase in the sulfamidase activity into the brain of injected MPS-IIIA mice, thus indicating that modified sulfamidase was able to efficiently cross the BBB. Moreover, an overall amelioration of brain pathology (including lysosomal storage, inflammation and autophagic stress) together with an improvement of behavioural abnormalities was observed in treated animals.
These data provide a proof of principle that modifying sulfamidase with domains that increase secretion efficiency and allow BBB transcytosis has promising therapeutic potential for the design of a low-invasive strategy to treat the brain pathology in MPS-IIIA.
However, we found some variability in the brain transduction of the chimeric sulfamidase, this indicating that besides the efficiency of ApoB-BD-mediated BBB transcytosis, other key factors may limit BBB crossing and/or brain transduction of the modified enzyme. The BBB molecular signature mediates the transcytosis process and may significantly change in pathological conditions and in response to different metabolic conditions. Therefore, we are now investigating on the presence of dissimilarities among the BBB signatures of treated MPS-IIIA mice and how/to what extent these changes influence the BBB crossing of the modified sulfamidase. We believe that these studies are important to improve the efficacy of our therapeutic approach.
In conclusion, our strategy will open new perspectives for clinical gene and enzyme replacement therapeutic protocols for MPS-IIIA as well as for other neurodegenerative LSDs caused by hydrolase defects.

ABSTRACT N. 4

TIGEM - Other Genetic Diseases		
Principal Investigator	**BRUNETTI PIERRI NICOLA**	
Telethon grant N.	**TGM11MT3**	
Total budget approved €	**410.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

GENE THERAPY FOR INBORN ERRORS OF HEPATOCYTE METABOLISM

Castello Raffaele (1), Borzone Roberta (1), d'Aria Stefania (1), Piccolo Pasquale (1), Annunziata Patrizia (1), Brunetti-Pierri Nicola (1,2)

(1) Telethon Institute of Genetics and Medicine - TIGEM, Via P. Castellino 111, 80131 Napoli; Tel. 081 6132361; Fax 081 5609877; E-mail: brunetti@tigem.it
(2) Department of Pediatrics, Federico II University of Naples, Italy

Helper-dependent adenoviral (HDAd) vectors are attractive for gene therapy because they can drive long-term expression of therapeutic genes and result in long-term phenotypic correction of multiple disease animal models. However, systemic high dose administration, required for efficient gene transfer to the liver, results in the activation of an acute inflammatory response with potentially severe and lethal consequences. We have previously developed an approach for vector delivery that resulted in improved therapeutic index of the vector and has proven to be successful in large animal models (i.e. nonhuman primates). This approach entails the use of balloon occlusion catheters to deliver HDAd preferentially to the liver. Based on the expression levels achieved using low vector doses, it is likely that clinically relevant benefit can be achieved in inborn errors of liver metabolism using low and clinically relevant vector doses. Primary hyperoxaluria type 1 (PH1), due to deficiency of the liver specific alanine:glyoxylate aminotransferase (AGT) gene, is an excellent disease candidate for clinical investigation of this gene therapy approach because of a favorable risk-benefit ratio, availability of direct measures of clinical benefit, and sufficient number of patient available for enrollment in a clinical trial. Towards this goal, we have generated preclinical data for liver-directed gene therapy of PH1 using HDAd vectors. We have shown that a single intravenous injection of an HDAd expressing the human AGT gene under the control of a liver-specific expression cassette results in long-term normalization of urinary oxalate excretion. Moreover, we have shown that an HDAd vector expressing a gene encoding an enzyme involved in glyoxylate detoxification results in reduction of urinary oxalate levels and kidney stone formation. In summary, our preclinical studies support the efficacy of gene therapy for treatment of PH1 and show that metabolic diversion achieved by gene over-expression is a novel therapeutic option for the treatment of PH1.

ABSTRACT N. 5

TIGEM - Other Genetic Diseases		
Principal Investigator	**DI BERNARDO DIEGO**	
Telethon grant N.	**TGM11SB1**	
Total budget approved €	**500.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

SYSTEMS BIOLOGY OF GENETIC DISEASES: ELUCIDATING GENE FUNCTION AND DRUG MODE OF ACTION

Gambardella Gennaro (1), Pagliarini Roberto (1), di Bernardo Diego (1,2)

(1) TIGEM, Via P. Castellino 111, 80131, Napoli, Italy - TIGEM, Via P. Castellino 111, 80131, Napoli, Italy; Tel. 0816132319; Fax 0816132351; E-mail:dibernardo@tigem.it
(2) Dipartimento Informatica e Sistemistica, Università degli Studi "Federico II", , Napoli, Italy

The objective of this project is to develop and apply Systems Biology approaches to gain a systems-level understanding of gene regulation at the transcriptional, post-transcriptional and post-translational level in specific tissues, and to relate regulation to metabolic function. Gene regulatory networks will be computationally reconstructed from heterogeneous large-scale experimental data including gene and microRNA expression profiles, protein-protein interactions and epigenetic data. The aim is to identify regulatory interactions including transcriptional regulation (Transcription Factor->target genes), post-transcriptional regulation (microRNA->target mRNAs) and post-translational regulation (kinase/phosphatase->target proteins). These results will be complemented by the development of a computational approach to identify candidate small-molecule compounds, which can specifically modulate these pathways, with the aim of identifying potential pharmacological treatments of genetic diseases. The methods will be applied to two study-cases: (i) identification of regulatory pathways specifically active in the human retina; (ii) identification of an integrated regulatory and metabolic network model of human hepatocytes to study inborn-errors of liver metabolism and to explore pharmacological therapies.
This project consists of three specific aims: each aim is divided into a methodological sub-aim and an applicative one. In Specific Aim1, we will develop a reverse-engineering approach based on Bayesian learning to reconstruct tissue-specific gene regulatory networks from gene expression profiles and using informative priors to capture known regulatory interactions from literature and other genome-scale experimental datasets. We will apply the method to reverse-engineer a human retina-specific gene regulatory network by generation and analysis of 50 gene expression profiles measured from samples obtained from normal human subjects. In Specific Aim 2, we will integrate reverse-engineered gene regulatory networks with genome-scale metabolic network models in order to identify tissue-specific metabolic pathways and their "master regulators" at the transcriptional and post-transcriptional level. We will reverse-engineer a hepatocyte-specific gene regulatory network integrated to a hepatocyte-specific metabolic network model to study Primary Hyperoxaluria Type 1, an inborn-error of liver metabolism due to the deficiency of the AGT enzyme, which causes accumulation of oxalate leading to renal failure. In Specific Aim 3, we will build on our previous work to further develop a computational approach to identify drug mechanism of action by integrating analysis of transcriptome changes following treatment with small molecules to tissue-specific gene regulatory networks. The computational approach will then be used to identify small-molecules with a therapeutic effect in Primary Hyperoxaluria Type 1.

ABSTRACT N. 6

TIGEM - Other Genetic Diseases		
Principal Investigator	**POLISHCHUK ROMAN**	
Telethon grant N.	**TGM11CB4**	
Total budget approved €	**214.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

PATHOGENESIS OF WILSON DISEASE: MOLECULAR MECHANISMS OF ATP7B TRAFFICKING IN THE MAINTENANCE OF COPPER HOMEOSTASIS

Chesi Giancarlo (1), Polishchuk Elena (1), Parashuraman Raman (1,2), Hegde Ramanath (1,2), Iacobacci Simona (1), Concilli Mafalda (1), Di Bernardo Diego (1), Luini Alberto (1,2), Polishchuk Roman (1)

(1) Telethon Institute of Genetics and Medicine - Via Pietro Castellino, 111, 80131, Naples, Italy, Tel. +39 081 6132336, Fax +39 081 5609877, E-mail: polish@tigem.it
(2) Institute of Protein Biochemistry, CNR, Naples, Italy

Wilson disease is an inherited autosomal recessive disorder of copper metabolism that is characterized by hepatic cirrhosis and neuronal degeneration caused by marked impairment in the biliary copper (Cu) excretion. Wilson disease arises from mutations in the ATP7B gene, which encodes the Cu-translocating ATPase, ATP7B. This ATP7B has a key role in hepatic copper excretion by virtue of its ability to transport Cu across the cellular membrane at the expense of ATP hydrolysis. A unique feature of ATP7B that is integral to this function is its ability to sense and respond to intracellular Cu levels. This response is manifested through its Cu-regulated trafficking from the trans-Golgi network to the appropriate cellular membrane domain, where ATP7B can then eliminate excess Cu from the cell. This property of ATP7B is affected by Wilson-disease-causing mutations that, therefore, result in toxic Cu accumulation in hepatocytes.
Most frequent ATP7B disease-associated mutations (R778L and H1069Q) result in aberrant protein products that are strongly mistargeted from the Golgi towards the endoplasmic reticulum (ER). Although they still exhibit residual Cu translocating activity, these ATP7B mutants fail to traffic to the biliary surface domain of hepatocytes, where Cu excretion takes place. This results in toxic Cu accumulation in hepatocytes. Thus, our main objective was to identify molecular targets for correction of the localization and trafficking of these disease-causing ATP7B mutants.
Given that properties of the ATP7B mutants are similar to those of main cystic fibrosis-causing mutant CFTR∆508F, clinically tested correctors of CFTR∆508F were analyzed for their ability to rescue aberrant ATP7B proteins. Treatment with CFTR∆508F correctors partially restored regular Golgi localization and Cu-induced trafficking of H1069Q mutant form. Further bioinformatics analysis, based on the evaluation of gene expression behavior in CFTR∆508F corrector-treated cells, revealed a specific phosphorylation cascade as a potential drug target for correction of both CFTR and ATP7B mutants. In this context, we found that inhibitors of two kinases induced very efficient recovery of both H1069Q and R778L mutants toward the Golgi as well as supported their trafficking in response to increasing Cu. Taken together our results provide a basis for novel therapeutic approach to combat Wilson disease.

ABSTRACT N. 7

TIGEM - Other Genetic Diseases		
Principal Investigator	**AURICCHIO ALBERTO**	
Telethon grant N.	**TGM11MT6**	
Total budget approved €	**300.000**	
Centres: **3**	*Duration (yrs):* **5**	*Starting year:* **2011**

TOWARDS CLINICAL TRIALS FOR AAV-MEDIATED EYE- AND LIVER-DIRECTED GENE THERAPY

Ferla Rita (1,2), Castello Raffaele (1), Marrocco Elena (1), Claudiani Pamela (1,2), Borzone Roberta (1), Gargiulo Annagiusi (1), Fontanella Bianca (1), Haskins Mark (3), Surace Enrico Maria (1), Brunetti-Pierri Nicola (1,2), Auricchio Alberto (1,2)

(1) Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. 0816132229, Fax 0815790919, E-mail: auricchio@tigem.it
(2) Dept. of Pediatrics, "Federico II" University, Naples, Italy
(3) Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

Gene transfer with adeno-associated viral (AAV) vectors for inherited diseases is obtaining the first evidences of clinical success. Important achievements have been obtained in three independent clinical trials for an inherited form of childhood blindness (Leber congenital amaurosis type 2, LCA2), to one of which we have made a major contribution, and in a clinical trial for hemophilia B in which patients have received intravascular administrations of AAV2/8. We want to build upon these promising results and on our long-standing experience in the development and preclinical applications of AAV vectors to rapidly move AAV-mediated gene transfer from bench to bedside for three ideal disease targets.
We decided to focus on LCA type 4 (LCA4) and on two inborn errors of metabolism, mucopolysaccharidosis VI (MPS VI) and primary hyperoxaluria type 1 (PH1). All three disorders are inherited as recessive traits and are excellent candidates for gene therapy: LCA4, a blinding condition similar to LCA2; MPS VI, a lysosomal storage disease without central nervous system involvement; and PH1, due to a defect in a liver-specific enzyme resulting in systemic accumulation of a toxic metabolite. For these disorders, there is currently no treatment or when available, therapies are largely unsatisfactory. In our laboratories, we have recently produced or are currently generating preclinical evidence of the efficacy of AAV-mediated gene transfer in animal models of these disorders. For all three diseases, we are using AAV2/8, which is the most efficient serotype for transduction of both retinal photoreceptors and hepatocytes. Our preliminary results show the tremendous potential of this vector which results in long-term therapeutic levels of expression in both eye- and liver-directed gene therapy.
The overall objective of this program is to complete the preclinical studies of efficacy and safety of AAV-mediated gene transfer required to rapidly move these three projects into the clinic. These investigations include AAV vector dose-response studies in rodents and, whenever available, in larger animal models (MPS VI cats and normal pigs), optimization of the expression cassettes, and comparison of single stranded vs. self complementary AAV. Concomitantly, in collaboration with the Departments of Ophthalmology and Pediatrics in Naples, Italy, we are characterizing at the clinical and molecular levels a large series of patients with LCA4, MPS VI, and PH1. We rely on a newly established TIGEM Regulatory Office to file the documents required to perform clinical trials for these disorders. This growing infrastructure and close collaborations with the clinicians uniquely position us to effectively move from bench to bedside our gene therapy research for three ideal disease targets using AAV vectors to target the retina and liver.

ABSTRACT N. 8

TIGEM - Other Genetic Diseases		
Principal Investigator	**SURACE ENRICO**	
Telethon grant N.	**TGM11MT2**	
Total budget approved €	**290.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

EFFICACY AND SAFETY OF TRANSCRIPTIONAL REPRESSORS AS BIOTHERAPEUTICS FOR THE TREATMENT OF AUTOSOMAL DOMINANT RETINITIS PIGMENTOSA (ADRP)

Botta Salvatore (1), Mussolino Claudio (1), Marrocco Elena (1), de Prisco Nicola (1), Giunti Massimo (3), Della Corte Michele (2), Rossi Settimio (2), Bacci Maria Laura (3), Simonelli Francesca (2), Surace Enrico Maria (1)

(1) Telethon Institute of Genetics and Medicine (TIGEM) - Via Pietro Castellino 111, 80131, Napoli, Tel. 0816132334, E-mail: surace@tigem.it
(2) Dept of Ophthalmology, Second University of Naples
(3) Dept of Veterinary Morphophysiology and Animal Production (DIMORFIPA), University of Bologna

We recently demonstrated that adeno-associated (AAV) vector somatic gene delivery of zinc-finger based transcriptional repressors is capable of robust down-regulation of an endogenous expressed gene, the photoreceptor-specific human Rhodopsin (RHO). We showed that a selected artificial transcriptional repressor delivered by AAV vector to diseased photoreceptors impact the fate of photoreceptor disease progression in a mouse model of an inherited form of retinal degeneration, autosomal dominant Retinitis Pigmentosa (adRP). The transcriptional repressor strategy was designed to overcome genetic heterogeneity (mutational independent transcriptional repression: silencing of both affected and unaffected genes loci, to be then coupled with somatic gene replacement; repression–replacement strategy), thus providing the generation of a unique therapeutic means to treat distinct mutations affecting a single gene. This strategy has particular relevance for highly mutated genes such as RHO, in which more than 150 mutations have been described. However, the transcriptional repressor that we thus far developed has specificity for the human RHO promoter, thus hampering its proper extensive testing in distinct animal species.
The objective of the project is to design novel artificial transcription repressors (based on both zinc finger-and Transcription activator–like (TAL) technology platforms) universally functional across species, with the goal to fully characterize their mode of action, safety and efficacy in relevant animal models of retinal diseases.

ABSTRACT N. 9

TIGEM - Other Genetic Diseases		
Principal Investigator	**BANFI SANDRO**	
Telethon grant N.	**TGM11SB2**	
Total budget approved €	**500.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

MicroRNA-REGULATED GENE NETWORKS IN THE RETINA

Conte Ivan (1), Karali Marianthi (1), Carrella Sabrina (1), Pizzo Mariateresa (1), Avellino Raffaella (1), Barbato Sara (1), Meola Nicola (1), Marrocco Elena (1), Surace Enrico Maria (1), Banfi Sandro (1,2)

(1) Telethon Institute of Genetics and Medicine (TIGEM), Via Pietro Castellino 111, 80131 Naples, Italy, Tel. +39-0816132228, Fax +39-0815609877, E-mail: banfi@tigem.it
(2) Department of Biochemistry, Biophysics and General Pathology, Second University, Naples

MicroRNAs (miRNAs) are a class of short non-coding RNAs that control the expression levels of their target genes by transcript degradation or translational inhibition. They exert important roles in the differentiation of many tissues and organs and are emerging as both players in the pathogenesis of diseases and possible therapeutic targets in human disorders. We are interested in the elucidation of the functional role of miRNAs in the retina, which represents the tissue target of a number of inherited forms of blindness including retinitis pigmentosa (RP). To achieve this goal, we are pursuing two parallel approaches:
1) we are developing algorithms, based on co-expression analysis, to reconstruct miRNA-regulated gene networks. In particular, we recently developed the Co-expression Meta-analysis of miRNA Targets (CoMeTa) tool that can effectively assign high-resolution biological functions to miRNAs and provide a comprehensive, genome-scale analysis of human miRNA regulatory networks [Gennarino VA, et al. Identification of microRNA-regulated gene networks by expression analysis of target genes. Genome Res 2012].
2) we are carrying out a detailed functional characterization of the miR-181 and of miR-204/211 subfamilies of miRNAs. Concerning the latter, we previously demonstrated, by using gain- and loss-of-function approaches in the medaka fish [Oryzias latipes (ol)] model organism, that alteration of miR-204 activity has a significant impact on multiple aspects of eye differentiation and function. In particular, morpholino-mediated ablation of miR-204 expression resulted in an eye phenotype characterized by microphthalmia and altered dorso-ventral (D-V) patterning of the retina, which causes optic coloboma [Conte I, et al. miR-204 is required for lens and retinal development via Meis2 targeting. Proc Natl Acad Sci U S A 2010, 107:15491-15496]. Additional evidence now suggests that miR-204 and its highly related mammalian paralog miR-211 has a significant impact on proper retinal axon guidance during eye development. Finally, we assessed, both in medaka fish and in mouse, that miR-204/211 play a crucial role also in the retina being required for correct photoreceptor cell differentiation and survival. In particular, we found that the inactivation of these two miRNAs determine a notable decrease of cone photoreceptors, as determined at both the molecular (immunofluorescence) and functional (electroretinogram) levels. Altogether our data indicate that miR-204 and miR-211 are key regulators of eye function in vertebrates and must be regarded as candidates for human eye disorders.

ABSTRACT N. 10

TIGEM - Other Genetic Diseases		
Principal Investigator	**AURICCHIO ALBERTO**	
Telethon grant N.	**TGM11MT1**	
Total budget approved €	**450.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

OVERCOMING THE CHALLENGE OF LARGE GENE TRANSFER FOR THE THERAPY OF INHERITED PHOTORECEPTOR DISEASES

Trapani Ivana (1), Colella Pasqualina (1), Puppo Agostina (1), Bartolomeo Rosa (1), Sommella Andrea (1), Iodice Carolina (1), Cesi Giulia (1), De Simone Sonia (1), Auricchio Alberto (1,2)

(1) Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. 0816132229, Fax 0815790919, E-mail: auricchio@tigem.it
(2) Dept. of Pediatrics, "Federico II" University, Naples, Italy

Inherited retinal diseases (IRDs) cause blindness in over 250,000 individuals in Europe. The majority are due to mutations in genes expressed in photoreceptors (PR) in the retina. We have recently demonstrated the safety and efficacy of gene therapy for IRDs in patients with Leber congenital amaurosis (LCA). One of the major limitations to extend this clinical success to other blinding conditions is that many are caused by mutations in genes with large (> 5 kb) coding sequences that exceed the cargo capacity of the most efficient gene transfer vector for PR, the adeno-associated virus (AAV). Conversely, vectors with larger cloning capacity like lentiviral (LV) or helper-dependent adenoviral (HD-Ad) vectors have poor PR tropism.
This project aims at overcoming the challenge of large gene delivery to PR. We propose to either expand AAV cargo capacity or to identify/modify LV and HD-Ad vectors with improved PR transduction efficiency. We plan to expand AAV cargo capacity by generating 2-split AAV vectors each containing one of 2 halves of a large gene which is reconstituted upon AAV intermolecular concatemerization in the nucleus of target cells. In parallel, we will screen a series of existing LV pseudotypes and adenoviral (Ad) vector serotypes for their ability to transduce PR. We will compare the efficiency of the three vector platforms to transduce murine and porcine PR. The platform with highest PR transduction efficiency will then be used to correct the retinal phenotype of murine models of common severe IRDs due to mutations in large genes.
Overcoming the challenge of large gene transfer will allow to cure common PR-specific diseases which are currently not amenable to treatment.

ABSTRACT N. 11

TIGEM - Other Genetic Diseases		
Principal Investigator	**LUINI ALBERTO**	
Telethon grant N.	**TGM11CB5**	
Total budget approved €	**229.100**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

DEVELOPING A RATIONAL BASIS FOR PHARMACOLOGICAL CORRECTION OF THE D508-CFTR FOLDING–TRAFFICKING DEFECTS

Parashuman Raman, Hegde Ramanath, Ciciriello Fabiana, Carissimo Anna Maria, Mutarelli Margherita, Di Bernardo Diego, Luini Alberto

Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. +39.081.6132535, Fax +39.081.6132277, E-mail: luini@tigem.it

Cystic Fibrosis (CF) is caused by mutations associated with the chloride channel, CF transmembrane regulator (CFTR), present in the apical plasma membrane of epithelial cells lining the lung, pancreas and other organs. The majority of the patients carry a mutation leading to a loss of the phenylalanine residue at position 508 (DF508-CFTR) of the protein that leads to its misfolding and subsequent intracellular retention in the endoplasmic reticulum (ER), where it is degraded. Numerous studies have identified the molecular mechanisms involved in the folding, degradation and trafficking (or, collectively, the proteostasis) of DF508-CFTR. Here we propose to utilize systems biological methods to identify the regulatory mechanisms that control the proteostasis of DF508-CFTR. We then plan to concentrate on the elucidation of these mechanisms (e.g., phosphorylation networks, ubiquitination networks, transcriptional networks etc.) and in particular to identify those key components that can favor the exit of the mutant CFTR from the ER to the plasma membrane. Regulatory modules, and especially the phosphorylation and ubiquitination networks, are druggable and so provide a possible therapeutic opportunity. Our pilot studies based on generating transcriptional profiles induced by drugs that correct the proteostasis defect of DF508-CFTR, and on the processing of these data together with the available published data on DF508-CFTR proteostasis, have led us to the identification of many novel regulatory pathways controlling DF508-CFTR folding/trafficking. Inhibitors of one such pathway have already shown promising evidence for escape of mutated-CFTR from the ER to a level that is achieved by currently available correctors. We also find that the regulatory pathways synergize with one another and the simultaneous modulation of several regulatory pathways improves level of correction by 2-3 fold over the currently achieved levels.

ABSTRACT N. 12

TIGEM - Other Genetic Diseases		
Principal Investigator	**DE MATTEIS MARIA ANTONIETTA**	
Telethon grant N.	**TGM11CB1**	
Total budget approved €	**300.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

DISSECTING THE MOLECULAR MECHANISMS UNDERLYING ENDOCYTIC DYSFUNCTIONS INDUCED BY MUTATIONS IN OCRL AND CLC5 TO IDENTIFY CORRECTORS FOR LOWE SYNDROME AND DENT DISEASE

Vicinanza Mariella (1), De Leo Giovanna (1), Santoro Michele (1), Marzolo MariaPaz (2), Levtchenko Elena (3), Di Campli Antonella (4), De Matteis Maria Antonietta (1)

(1) Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. 081.6132220, E-mail: dematteis@tigem.it
(2) Department of Cellular and Molecular Biology, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
(3) Department of Paediatrics, University Hospitals Leuven, Leuven, Belgium
(4) IBP, CNR, Naples

The inherited renal Fanconi syndromes are a group of genetically heterogeneous disorders that are characterized by an impaired reabsorptive function of renal proximal tubular cells (PTCs), and, as a consequence, by the urinary loss of salts, bicarbonate, nutrients and low molecular weight (LMW) proteins leading to severe systemic implications, such as metabolic acidosis and vitD deficiency. The reabsorptive function of the PTCs relies on a complement of receptors, transporters and channels acting at the apical membrane of PTCs. In the case of LMW proteins and other nutrients, the "multiligand" receptor megalin captures ligands in the ultrafiltrate and, via clathrin-mediated endocytosis, internalizes and delivers them to deeper endocytic stations while megalin recycles back to the apical plasma membrane. Many of the ion transporters, including the Na+/H+ exchanger (NHE3) and NaPhosphate cotransporter NaPI3, undergo continuous cycling between the apical plasma membrane and the endosomes. Thus, the endocytic pathway is a key component of the reabsorptive apparatus of the PTCs. Therefore, not surprisingly, among the genes that are defective in Fanconi syndrome are those encoding these receptors and transporters but also endosomal proteins such as the PI45P2 5-phosphatase OCRL and the Cl-/H+ antiporter CLC5. However, the function of these proteins in endocytic membrane trafficking of PTCs is not fully defined. Mutations in CLC5 cause Dent disease (characterized by Fanconi syndrome with nephrocalcinosis) while mutations in OCRL are responsible for Lowe syndrome (characterized by renal Fanconi syndrome, congenital cataracts and central hypotonia with variable mental impairment) but can also cause Dent disease. This project is focused on studying the function of OCRL and CLC5 in membrane trafficking and signalling at the PTCs. The rationale is to exploit the overlap of the clinical outcome caused by mutations of the two genes to delineate the molecular networks regulating the trafficking and signalling pathways responsible for protein and salt reabsorption in PTCs. Importantly, once we have gained a deeper knowledge of the cell biology of these gene products we will exploit the system to look for correctors of their defects.

ABSTRACT N. 13

TIGEM - Other Genetic Diseases		
Principal Investigator	**FRANCO BRUNELLA**	
Telethon grant N.	**TGM11CB3**	
Total budget approved €	**284.100**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

OFD1 REGULATES BROAD PARACRINE SIGNALING BY CONTEXT-SPECIFIC PROTEASOMAL DEGRADATION OF SIGNALING MEDIATORS

Morleo Manuela (1), Liu Yangfan P (2), Massa Filomena (1), Amato Roberto (1), Oh Edwin (2), Tsai I-Chun (2), Lee Byung-Hoon (3), Finley Daniel (3), Di Bernardo Diego (1), Katsanis Nicholas (2), Franco Brunella (1,4)

(1) Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. 0816132207; E-mail: franco@tigem.it
(2) Center for Human Disease Modeling, Durham, USA
(3) Department of Cell Biology, Harvard Medical School, Boston
(4) Department of Pediatrics Federico II University, Naples

Cilia are microtubule-based organelles protruding from the cell surface of almost all mammalian cells and exert diverse motility and sensory function within the cell. Work from different groups revealed that cilia have crucial roles in cell signaling pathways and in maintaining cellular homeostasis. Ciliary dysfunction has been implicated in several disorders called "ciliopathies" including, among others, Oro-facio-digital type I (OFDI) syndrome. Thousands of proteins potentially involved in ciliary function have been identified. Recent reports clearly established that primary cilia are necessary for Sonic Hedgehog (Shh) signaling and other pathways. However, much remains to be determined on the biology and functions of this complex organelle of growing biomedical importance. OFD1 codifies for a basal body/centrosomal protein which has been shown to be crucial for cilia formation. We applied a system biology approach to provide a preliminary characterization of the Cilia/Centrosome Complex interactome: the analysis revealed that OFD1 is strongly "connected" to the components of the proteasome pathway. Moreover mass spectrometry experiments identified as putative OFD1 interactors different members belonging to the 19S regulatory particle of the 26S proteasome complex. We hypothesized that OFD1 could have a proteasome dependent role and thus mediate protein degradation. Indeed we demonstrated that impairment of proteasomal degradation leads to accumulation of components of the Sonic Hedgehog (Shh) pathway in Ofd1 deficient models. In addition, OFD1 depleted in vivo and in vitro models also display increased levels of components of the Notch, Wnt and NF-KB signaling pathways. Using a biochemical approach, we demonstrated that: a) loss of OFD1 perturbs the degradation of both ubiquitin-dependent and –independent proteasomal targets; b) OFD1 interacts with proteasomal subunits and c) loss of OFD1 leads to depletion of multiple regulatory subunits from the centrosomal proteasome. Interestingly, we observed that also mutant models for other ciliopathies such as Bardet-Biedl (BBS) syndromes, display loss of proteasomal degradation of signaling mediators. Consistently, we were able to ameliorate BBS and OFDI established Notch and Wnt signaling defects in vivo, by modulating proteasome subunits. Taken together, our data indicate that basal body-proteasome regulation is a common mechanism governing the regulation of paracrine signaling, and suggest that the modulation of the proteasome might be of clinical benefit to some ciliopathy patients.

ABSTRACT N. 14

TIGEM - Other Genetic Diseases		
Principal Investigator	**DE MATTEIS MARIA ANTONIETTA**	
Telethon grant N.	**TGM11CB2**	
Total budget approved €	**300.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

DEFINING THE CELLULAR AND MOLECULAR BASIS OF THE SPONDYLOEPIPHYSEAL DYSPLASIA TARDA AND IDENTIFICATION OF TARGETS FOR PHARMACOLOGICAL INTERVENTION

Venditti Rossella (1), Zappa Francesca (1), Santoro Michele (1), Zelante Leopoldo (2), Malhotra Vivek (3), VerTel.Barbara (4), De Matteis Maria Antonietta (1)

(1) Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, Naples, Italy, Tel. 081.6132220, E-mail: dematteis@tigem.it
(2) Medical Genetics Unit, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy
(3) Department of Cell and Developmental Biology, Centre de Regulacio Genomica, Barcelona, Spain and Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain
(4) Department of Cell Biology and Anatomy, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, USA

We propose to study the cellular pathogenesis mechanism of spondyloepiphyseal dysplasia tarda (SEDT, OMIM: 313400), a rare recessive X-linked osteochondrodysplasia caused by mutations in the sedlin gene (TRAPPC2, OMIM: 300202), as a means to unravel the trafficking machinery that is involved in the transport of one of the most abundant and largest secretory cargoes of chondrocytes, type II procollagen (PCII). Indeed, we have obtained important preliminary results indicating that sedlin is specifically required for the exit of PCII from the ER, thus representing a first line of investigation that we can exploit to reconstruct the entire machinery involved in the transport of this cargo that is special in terms of both its size and abundance in chondrocytes.

We propose to combine information from studies in mammalian cells, including the cells most relevant for the disease (chondrocytes), with those from studies of the yeast counterpart of sedlin, Trs20, to dissect the role of this protein in membrane trafficking in general and in chondrocytes in particular. Importantly, we aim to use yeast cell biology and genetic studies to search for genes that can suppress the defect of Trs20 dysfunction that might provide leads for the identification of modifier genes in mammals. Once we acquire a more accurate knowledge of the function of this protein, we will exploit it to search for small molecules or drug targets that can correct the cellular defect in PC transport. The active targets will be validated in appropriate animal models that we propose to develop.

SAN RAFFAELE TELETHON INSTITUTE FOR GENE THERAPY

ABSTRACT N. 15

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**AIUTI ALESSANDRO**	
Telethon grant N.	**TGT11A01**	
Total budget approved €	**576.800**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

Sauer Aisha V (1), Brigida Immacolata (1), Carriglio Nicola (1,2), Jofra Hernandez Raisa (1), Giannelli Stefania (1), Ferrua Francesca (3), Di Lorenzo Biagio (1), Sanvito Francesca (4), Poliani Pietro L (5), Traggiai Elisabetta (6), Carlucci Filippo (7), Van der Burg Mirjam (8), Meffre Eric (9), Aiuti Alessandro (1,2)

(1) Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, Milan, Italy; Tel. 02-26434435, E-mail: a.aiuti@hsr.it
(2) University of Rome "Tor Vergata", Rome, Italy
(3) Pediatric Immunohematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milano, Italy
(4) Pathology Unit, Department of Oncology, San Raffaele H Scientific Institute, Milan, Italy
(5) Department of Pathology, University of Brescia, Brescia, Italy
(6) Second Division of Pediatrics, IRCCS, Institute G. Gaslini, Genova, Italy
(7) Department of Internal Medicine, Endocrine-Metabolic Sciences and Biochemistry, University of Siena, Italy
(8) Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
(9) Department of Immunobiology, Yale University School of Medicine, New Haven, USA

Adenosine deaminase (ADA)-SCID is characterized by impaired lymphocyte development and function, as well as systemic organ damage due to metabolic toxicity. Particularly ADA-deficient patients with late-onset forms and after enzyme replacement therapy (PEG-ADA) are known to manifest immune dysregulation, but autoimmunity was recently also observed in patients after hematopoietic stem cell gene therapy (HSC-GT). Our aim is to acquire new information on the autoimmune manifestations of this disease and to investigate the ability of different treatments to correct them.
Adenosine acts as anti-inflammatory mediator on the immune system and has been described in regulatory T cell (Treg)-mediated suppression. We obtained evidence that adenosine, accumulating in the absence of ADA and its excessive turn-over by PEG-ADA interfere with Treg function. Tregs isolated from PEG-ADA treated patients are reduced in number and show decreased suppressive activity, whereas they are corrected after gene therapy. PEG-ADA treated mice developed multiple autoantibodies and hypothyroidism in contrast to mice treated with bone marrow transplantation or gene therapy. Tregs isolated from PEG-ADA treated mice lacked suppressive activity, suggesting that this treatment interferes with Treg functionality. The observed loss of function in ADA-deficient Tregs provides new insights into a predisposition to autoimmunity and the underlying mechanisms causing defective peripheral tolerance in ADA-SCID.
We also assessed if B-cell development and function occur properly in ADA-deficiency. An in-depth characterization of bone marrow (BM) B-cell development in ADA-SCID patients showed a block at the preB1 cell stage in untreated patients that is overcome after treatment with PEG-ADA or HSC-GT. In the periphery, transitional B cells accumulate under PEG-ADA and normalize progressively after HSC-GT. The strongest selective advantage for ADA-transduced B cells was observed at the transition from immature to naïve B cells.
To assess whether ADA deficiency affects the establishment of B cell tolerance, we tested the reactivity of recombinant antibodies isolated from single B cells of ADA-SCID patients before and after HSC-GT. We found that before HSC-GT, B cells from ADA-SCID patients contained more autoreactive and ANA-expressing clones, indicative of defective central and peripheral B-cell tolerance checkpoints. Strikingly, after HSC-GT, ADA-SCID patients displayed quasi-normal early B cell tolerance. Patients under PEG-ADA showed impaired B cell proliferative responses after BCR/TLR triggering, which normalized in patients treated with HSC-GT. Overall, our data indicate ADA plays an essential role in controlling autoreactive B cell counterselection by regulating BCR and TLR functions. Moreover, our findings confirm the efficacy of HSC-GT in restoring both B-cell tolerance and function.

ABSTRACT N. 16

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**AIUTI ALESSANDRO**	
Telethon grant N.	**TGTGSK03**	
Costs incurred €	**1.755.700**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

CLINICAL TRIAL OF HEMATOPOIETIC STEM CELL GENE THERAPY FOR WISKOTT-ALDRICH SYNDROME

Scaramuzza Samantha (1), Biasco Luca (1), Ferrua Francesca (2), Cicalese Maria Pia (2), Giannelli Stefania (1), Castiello Maria Carmina (1,3), Evangelio Costanza (2), Assanelli Andrea (2), Casiraghi Miriam (2), Bosticardo Marita (1), Rizzardi Paolo (4), Finocchi Andrea (5), Metin Ayse (6), Banerjee Pinaki P (7), Orange Jordan S (7), Biffi Alessandra (1,2), Villa Anna (1,8), Ciceri Fabio (9), Roncarolo Maria Grazia (1,2,3), Naldini Luigi (1,3), Aiuti Alessandro (1,2,5)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milan, Italy, Tel. +390226434671, Fax +390226434668, E-mail: alessandro.aiuti@hsr.it
(2) Pediatric Immuno-hematology and Bone Marrow Transplant, Scientific Institute HS Raffaele, Milan, Italy
(3) Università Vita-Salute San Raffaele, Milan, Italy
(4) MolMed SpA, Milan, Italy
(5) University of Rome "Tor Vergata", Rome, Italy
(6) Ankara Dispaki Children's Hospital, Ankara, Turkey
(7) Texas Children's Hospital, Baylor College of Medicine, USA
(8) IRGB-CNR, Milan, Italy
(9) Hematology and Bone Marrow Scientific Institute HS Raffaele, Milan, Italy

Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterized by thrombocytopenia, infections, autoimmunity and lymphomas. In patients lacking a compatible hematopoietic stem/progenitor cell (HSPC) donor, mismatched allogeneic transplantation or gene therapy with gamma-retroviral vectors provided clinical benefit but resulted in transplant complications and genotoxicity of the vector, respectively. We adopted a new protocol of HSPC gene therapy based on a self-inactivating lentiviral vector (LV) expressing the WAS protein under its endogenous promoter combined with reduced intensity conditioning (anti-CD20, busulfan and fludarabin). Three patients were treated with autologous bone marrow (BM) CD34+ cells transduced with highly purified lentiviral vector. The first patient received also mobilized peripheral blood transduced CD34+ cells to reach an adequate cell dose. Transduction of clonogenic progenitors was highly efficient (94.3 ± 5.3%), with a mean VCN/genome in bulk CD34+ cells of 2.1 ± 0.6. We then evaluated the engraftment of transduced cells in bone marrow and peripheral blood (PB) lineages and patients' immune functions. A robust multilineage engraftment of gene corrected cells was observed in the PB and BM at 1-1.5 years after treatment. Molecular tests showed the presence of gene modified cells in BM clonogenic progenitors (25-50%), BM myeloid lineages (VCN range: 0.29-0.78), PB granulocyte (VCN: 0.56±0.15) and lymphocytes (VCN range: 1.05-2.29) at the latest follow-up. WASp expression was detected in platelets, monocytes and at higher levels in lymphoid cells, as assessed by flow cytometric analyses. Proliferative responses to anti-CD3, NK cell cytotoxic activity, immune synapsis formation and Treg suppressive function were normalized after gene therapy. All patients are currently clinically well, independent from platelet transfusions, free from eczema and severe infections. High-throughput sequencing of vector integration sites after transplant demonstrated a highly polyclonal haematopoiesis. Importantly, we were able to directly compare the LV and gamma-retroviral vector insertion profiles in cells from gene therapy treated patients with the same disease

background. This analysis highlighted significant differences in the genomic distribution of vector insertions between the two trials and showed that the LV gene therapy protocol did not induce in vivo selection of integrations near cancer genes or aberrant clonal expansions. The current clinical follow (1.4-2.4 years) and the cumulative data derived from other safety monitoring tests are consistent with an improved safety of LV gene therapy. In conclusion, LV-based HSPC gene therapy provides a new treatment option for WAS and, conceivably, other genetic blood disorders.

ABSTRACT N. 17

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**VILLA ANNA**	
Telethon grant N.	**TGT11A02**	
Total budget approved €	**533.700**	
Centres: **3**	*Duration (yrs):* **5**	*Starting year:* **2011**

CELLULAR AND MOLECULAR BASES OF AUTOIMMUNITY IN WISKOTT-ALDRICH SYNDROME

Castiello Maria Carmina (1,2), Catucci Marco (1), Pala Francesca (1), Prete Francesca (3), Benvenuti Federica (3), van der Burg Mirjam (4), Meffre Eric (5), Bosticardo Marita (1), Villa Anna (1,6)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), - Via Olgettina 58, Milan, Italy, Tel. 0226435273, E-mail: anna.villa@hsr.it
(2) Vita-Salute San Raffaele University, Milan, Italy
(3) International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149, Trieste, Italy
(4) Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
(5) Department of Immunobiology, Yale University School of Medicine, New Haven, CT, USA
(6) IRGB, Milan Unit, CNR

Wiskott-Aldrich Syndrome (WAS) is an X-linked primary immunodeficiency caused by mutations in the gene encoding for WAS protein (WASp), a regulator of cytoskeletal reorganization also involved in signal transduction of hematopoietic cells. WAS is characterized by microthrombocytopenia, eczema, infections and high rate of autoimmunity and tumors. The mechanisms underlying the occurrence of autoimmune manifestations have not been clearly described yet. Since the role of B cells in the pathogenesis of autoimmune diseases, we studied the B cell development at phenotypical and molecular levels in a cohort of 22 WAS pediatric patients. In all patients analyzed, transitional B cells were expanded, as observed in other immunodeficient conditions or autoimmune diseases. The increased usage of J-kappa 5 gene in WAS transitional B cells potentially suggests that receptor editing may not be properly regulated in developing B cells. We also found increased frequencies of CD21low B cells, which are expanded in SLE and RA and are known to be enriched in autoreactive clones. WAS B cells are characterized by a low expression of complement receptor 1, which could contribute to a chronic inflammatory state favoring breakdown of peripheral tolerance. Finally, WAS patients show a defective maturation of memory B cells showing a reduced in vivo proliferation and somatic hypermutation, in particular in T cell independent B cell subpopulations. Our data show that WASp-deficiency affects critical stages of central and peripheral B cell differentiation thus impairing humoral immune response and favoring the presence of autoreactive B cell populations in WAS patients. Next we analyzed plasmacytoid dendritic cells (pDCs) compartment shown in several autoimmune diseases contribute to breakage of tolerance. To investigate a possible contribution of altered pDC homeostasis to the pathophysiology of autoimmune manifestation in WAS, first we incubated pDCs isolated from healthy donors (HDs) with sera derived from WAS patients or HDs and we observed higher induction of IFN-a production by WAS sera, as compared to HDs and similar to the induction by SLE and Juvanile Arthritis sera. We observed increased expression of three IFN-a genes and five type-I IFN-induced genes as compared to age-matched HDs. In keeping with previous observations in SLE, we found a reduction in the frequency of pDCs in the PBMC of 15 pediatric WAS as compared to HDs. Next we evaluated the capacity of WAS pDCs to produce IFN-a in response to an agonist of TLR9 and we found that the relative IFN-a production by WAS patients was significantly higher compared to HDs. Overall these data strongly support the conclusion that the WAS pDCs/IFN-alpha axis can be altered in WAS patients and that elevated type-I interferon may contribute to pathogenesis of autoimmune manifestations.

ABSTRACT N. 18

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**VILLA ANNA**	
Telethon grant N.	**TGT11A03**	
Total budget approved €	**512.600**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

THYMIC AND PERIPHERAL IMMUNE RECONSTITUTION IN A MOUSE MODEL OF OMENN SYNDROME UPON ANTI-CD3EPSILON MAB TREATMENT

Marrella Veronica (1), Poliani Luigi (2), Fontana Elena (2), Grassi Fabio (3), Villa Anna (1,4)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), - Via Olgettina 58, Milan, Italy, Tel. 0226435273, E-mail: anna.villa@hsr.it
(2) Pathology Department, University of Brescia
(3) IRB, Bellinzona, Switzerland
(4) IRGB, Milan Unit, CNR

Omenn syndrome (OS) is an atypical primary immunodeficiency with autoimmune-like manifestations due to activated T cells infiltrating target organs.
The generation of a murine model carrying the Rag2 R229Q mutation previously described in several patients, represents an important tool to further understand the molecular and cellular pathophysiology of OS and to test alternative therapeutic approaches for patients whose unique available therapy is hematopoietic stem cell transplant (HSCT) from genotipically identical sibling donor.
Impaired recombinase activity reduces the expression of the pre-T cell receptor complex in immature thymocytes thus preventing the normal development of the thymic epithelial component. Thymic medulla plays a crucial role in the establishment and maintenance of central immune tolerance by eliminating self-reactive T cell clones and developing natural regulatory T cells. The Rag2R229Q mice show a profound disorganization in the organ architecture with reduced expression of autoimmune regulator (Aire) which governs thymic selection. These alterations, also described in patients, suggest that thymic defect together with low Rag activity are responsible for escape of auto reactive T cells clones causing peripheral organ damage. We have analyzed in details epithelial thymic defect in Rag2R229Q mice by means of FACS analysis and real-time PCR showing that thymic medullary compartment is severely compromised with a clear impairment in the maturation that can justify the reduced expression at mRNA level of Aire and Tissue specific antigens.
Since anti-CD3epsilon monoclonal antibody (mAb) treatment is known to promote thymic expansion and medulla maturation in Rag2-/- mice, we investigated its efficacy to address the relevance of the crosstalk between thymocytes and epithelial cells in preventing autoimmunity in Rag2R229Q mouse model. We have treated Rag2R229Q newborns with two doses of anti-CD3epsilon and analysed the effect of the administration on thymus and peripheral T cell compartment after two months. A significant improvement in the thymic epithelial compartment with clear medulla maturation was observed, although Aire expression was not induced by the treatment. In the peripheral compartment T cells show a reduction in the expression of activation markers, a reduced production of pro-inflammatory cytokines and a lower infiltration capacity. Indeed target organs were devoid of lymphocyte infiltrates leading to a general amelioration of the pathological picture. These findings indicate that improving the epithelial thymic component prevents the detrimental behaviour of the cell-autonomous RAG defect, and provide important therapeutic proof of concept for future clinical applications of anti-CD3epsilon mAb treatment in forms of severe combined immunodeficiency (SCID) characterised by poor thymus function and autoimmunity.

ABSTRACT N. 19

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**BACCHETTA ROSA**	
Telethon grant N.	**TGT11A04**	
Total budget approved €	**946.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

CELL/GENE TRANSFER BASED THERAPIES FOR IPEX SYNDROME AND FOXP3-GENE INDEPENDENT DISEASES WITH IMMUNE DYSREGULATION

Passerini Laura (1), Santoni de Sio Francesca (1), Barzaghi Federica (1), Rossi Mel Eva (1,2), Sartirana Claudia (1), Gizzi Maria Concetta (1,7), Levings Megan K. (3), Lampasona Vito (4), Bosi Emanuele (5,6,7), Naldini Luigi (1,7), Roncarolo Maria G. (1,7), Bacchetta Rosa (1)

(1) San Raffaele Telthon Institute for Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132, Milan, Italy, Tel. 0226434669; Fax 0226434668; E-mail:rosa.bacchetta@hsr.it
(2) University of Rome Torvergata, Rome, Italy
(3) University of British Columbia, Vancouver, Canada
(4) Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, Milan, Italy
(5) Autoimmunity Section, Laboraf, Ospedale San Raffaele, Milan, Italy
(6) Diabetes Research Institute, San Raffaele Scientific Institute, Milan, Italy
(7) Vita-Salute San Raffaele University, Milan, Italy

Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a life-threatening autoimmune disease due to mutations in Forkhead box P3 (FOXP3), the master transcription factor for CD4+CD25+ regulatory T cells (Tregs) (Barzaghi, Front Immunol 2012), essential for controlling immune responses to autoantigens. FOXP3 mutations result in Tregs dysfunction and instability and in the increase of circulating Th17 cells (Passerini, JACI 2011). The only curative treatment is haematopoietic stem cell transplantation (HSCT), which is not available for all patients. Furthermore, since there is no clear genotype-phenotype correlation (Gambineri, JACI 2008), there are no prognostic indicators for disease severity.
We have been working (i) to develop alternative therapeutic strategies for the cure of IPEX syndrome, (ii) to investigate the etiology of IPEX-like syndrome, in which IPEX symptoms occur in the absence of FOXP3 mutations, and (iii) to identify biomarkers to evaluate response to therapy and for differential diagnosis of IPEX and IPEX-like syndromes.
(i) We previously demonstrated that transduction of human CD4+ T cells with lentivirus (LV) encoding for FOXP3 generates a population of Tregs (Allan, Mol Ther 2008). By means of LV, we over-expressed FOXP3 into T cells isolated from several IPEX patients. The resulting CD4FOXP3 population expressed Treg markers, was anergic, suppressed cytokine production, and displayed potent suppressive activity both in vitro and in vivo, in a model of xenogeneic graft-versus-host disease. These results indicate that patients' effector T cells could be converted into functional Tregs, thus demonstrating that FOXP3 gene transfer is feasible.
(ii) By analysing demethylation of the Treg-cell-Specific-Demethylated-Region (TSDR) in the FOXP3 locus, specific marker for stable Tregs, we have demonstrated that in IPEX-like patients the amount of peripheral Tregs is reduced (Barzaghi, J Autoimmunity 2012). Since the Treg suppressive function was not impaired, this demonstrates that a quantitative defect of Tregs per se could sustain autoimmunity.
(iii) The 75kDa USH1C protein (harmonin) (Kobayashi, Clin Exp Immunol 1998) and the 95 kDa villin protein (Kobayashi, Clin Immunol 2011) have been previously identified as targets of IPEX-associated autoantibodies. We provided evidence that anti-harmonin and, to a minor extent, anti-villin autoantibodies (HAA and VAA), measured by LIPS assays, are detectable in the sera of all IPEX patients, but absent in IPEX-like subjects, thus providing a sensitive and specific marker of IPEX. In addition, HAA and VAA hold a significant prognostic value: HAA peaked during acute phase, at the time of onset, and relapse of enteropathy, and declined at remission and after successful HSCT.
In conclusion, our studies pave the way for more expert diagnosis and rationale therapeutic approaches for IPEX and IPEX-like syndrome, and increase our knowledge of the biology of immune regulation.

ABSTRACT N. 20

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**BIFFI ALESSANDRA**	
Telethon grant N.	**TGTGSK02**	
Costs incurred €	**1.557.300**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

PHASE I/II CLINICAL TRIAL OF HEMATOPOIETIC STEM CELL GENE THERAPY FOR THE TREATMENT OF METACHROMATIC LEUKODYSTROPHY

Montini Eugenio (1), Lorioli Laura (1,2,3,4), Cesani Martina (1), Fumagalli Francesca (2,5), Plati Tiziana (1), Martino Sabata (5), Baldoli Cristina (7), Calabria Andrea (1), Canale Sabrina (2), Benedicenti Fabrizio (1), Vallanti Giuliana (8), Biasco Luca (1), Leo Simone (9), Kabbara Nabil (10), Zanetti Gianluigi (9), Rizzo William B. (11), Metha Nalini AL. (12), Cicalese Maria PIa (2,3), Casiraghi Miriam (2), Boelens Jaap J (13), Del Carro Ubaldo (5), Dow David J (12), Schmidt Manfred (14), Di Serio Clelia (4), Stupka Elia (15), Von Kalle Christof (14), Bordignon Claudio (4,8), Ciceri Fabio (3,16), Rovelli Attilio (17), Roncarolo Maria Grazia (1,2,3,4), Aiuti Alessandro (1,2,3,18), Sessa Maria (2,5), Naldini Luigi (1,4), Biffi Alessandra (1,2,3)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; Tel. 0226434678; Fax 0226434668; E-mail: a.biffi@hsr.it
(2) HSR-TIGET Pediatric Clinical Research Unit, San Raffaele Scientific Institute, Milan
(3) Pediatric Immunohematology, San Raffaele Scientific Institute, Milan, Italy
(4) Vita Salute San Raffaele University, Milan, Italy
(5) Neurology Unit, Department of Neurology, San Raffaele Scientific Institute, Milan
(6) Neuroradiology Unit, Head and Neck Department, San Raffaele Scientific Institute, Milan
(7) Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy
(8) Molmed S.p.A., Milan, Italy
(9) Distributed Computing Group, Center for Advanced Studies, Research and Development in Sardinia (CRS4), Pula, Italy
(10) Pediatric Hematology Oncology Division, Rafic Hariri University Hospital, Beirut, Lebanon
(11) Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE, USA.
(12) Molecular and Cellular Technologies, GlaxoSmithKline, Stevenage, United Kingdom
(13) Pediatric Blood and Marrow Transplantation Program, UMC Utrecht, Netherland
(14) Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, Heidelberg, Germany
(15) Center for Translational Genomics and BioInformatics, San Raffaele Scientific Institute, Milan, Italy
(16) Hematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute
(17) Bone Marrow Transplant Unit, MBBM Foundation, Pediatric Department, Milano-Bicocca University at San Gerardo Hospital, Monza, Italy
(18) University of Rome Tor Vergata

Metachromatic Leukodystrophy (MLD) is an autosomal recessive lysosomal storage disorder caused by Arylsulfatase A (ARSA) deficiency and leading to severe demyelination, neurodegeneration and premature death of the affected patients. Currently, no treatment can halt the progression of this devastating disease. According to preclinical data demonstrating the safety and efficacy of hematopoietic stem cell gene therapy in the animal model of the disease, and based on the experience we acquired on the natural clinical course of the disease and its instrumental and clinical monitoring, on March 2010 a clinical trial based on transplantation of autologous hematopoietic stem cells transduced with lentiviruses (LVs) encoding ARSA was approved by the Italian Regulatory Authorities. The clinical protocol foresees the enrollment of 6 late infantile (LI) and 2 early juvenile (EJ) patients, in pre- and, in the case of EJ patients, early-symptomatic stage, in order to provide them a reasonable expectation of clinical benefit. The study objectives are the evaluation of the safety of the treatment, related to the myeloablative conditioning regimen employed and to the use of LVs, and of its efficacy by measuring patients' motor abilities and demyelination occurring in the nervous system through the use of validated instrumental readouts.
Until now seven patients have been enrolled and treated. Six of them had a biochemical, molecular and familiar history compatible with a diagnosis of LI MLD and have been treated in a pre-symptomatic stage of their disease. Only one patient, with a disease onset compatible with the EJ form of the disease, was treated in an early symptomatic stage. Thus far, we can report a favorable outcome of the transplant procedure with a good bone marrow recovery and the short/medium-term safety of both the conditioning regimen and stem cell transduction with LVs in all the treated patients. Moreover, we report stable sustained ARSA gene replacement to nearly exhaustive levels in the reconstituted hematopoiesis of the patients, resulting in supra-normal ARSA activity throughout the hematopoietic lineages and its reconstitution in the cerebrospinal fluid, the latter thus far documented in the first three treated patients. These findings are associated with substantial therapeutic benefit. Indeed, at the follow-up of the first three late infantile treated patients performed after the expected symptoms' onset (as defined according

to disease onset in the affected older siblings) the disease had not appeared/progressed; furthermore they are rather experiencing a continuous motor and cognitive development, at odds with the natural disease course and their sibling anamnesis, and have a normal quality of life. These data are extremely encouraging, even if only the long-term follow-up of all the treated patients will confirm this favorable preliminary indication.

ABSTRACT N. 21

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**BIFFI ALESSANDRA**	
Telethon grant N.	**TGT11B01**	
Total budget approved €	**596.600**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

HSC GENE THERAPY FOR LSDs: UNDERSTANDING THE MODALITIES OF CELL REPLACEMENT IN THE LSD BRAIN FOR IMPROVING THERAPEUTIC EFFICACY

Capotondo Alessia (1), Milazzo Rita (1,2), Politi Letterio Salvatore (3,4), Cecere Francesca (1), Quattrini Angelo (5), Palini Alessio (6), Plati Tiziana (1), Montini Eugenio (1), Naldini Luigi (1,2), Biffi Alessandra (1)

(1) HSR-TIGET, San Raffaele Scientific Institute, Via Olgettina 58, Milano, Italy; Tel. 0226434678; Fax 0226434668; E-mail: a.biffi@hsr.it
(2) Vita-Salute San Raffele University, San Raffaele Scientific Institute, Milano, Italy
(3) Imaging Core, San Raffaele Scientific Institute, Milano, Italy
(4) Neuroradiology Unit, Head and Neck Department, San Raffaele Scientific Institute, Milano, Italy
(5) Division of Neuroscience, San Raffaele Scientific Institute, Milano, Italy
(6) Flow Cytometry Resource Analytical Cytology Technical Applications Laboratory, San Raffaele Scientific Institute, Milano, Italy
(7) Center of Biostatistics, Università Vita-Salute San Raffaele, San Raffaele Scientific Institute, Milano, Italy

From the impressive results originally obtained in Hurler syndrome, it was expected that Hematopoietic Cell Transplantation (HCT) could alleviate most Lysosomal Storage Disorders (LSDs). However, HCT was proven to provide diverse degree of benefit according to the disease, the involvement of the CNS and its stage at time of transplant and, in particular, it was proved to be ineffective in LSD patients with overt neurological symptoms or in those with early onset or aggressive infantile forms. In the previous funding period, we have addressed the issue of limited HCT efficacy by increasing the gene/enzyme dose in brain macrophages and microglia by means of gene transfer into the transplanted hematopoietic stem cells (HSCs). Based on the promising results obtained with this approach on the murine disease models, a clinical trial of HCS gene therapy is currently active in Metachromatic Leukodystrophy (MLD). However, a critical need exists also to enhance and fasten brain microglia turnover with donor cells following HCT in order to anticipate the time of clinical benefit and improve the efficacy of the transplant procedure. Despite in several reports the occurrence of progressive brain myeloid infiltration and microglia reconstitution by donor cells was demonstrated following hematopoietic stem and progenitor cell (HSPC) transplantation, the mechanisms of microglia reconstitution after the transplant are far from being understood and are still debated. Indeed, we investigated the modalities of microglia reconstitution after HSPC transplantation, tracking a kinetic of the myeloid infiltration into the brain, in basal conditions or after the administration of different preparative regimens. By studying wild type and LSD mice at diverse time-points following HCT we showed the occurrence of a short-term wave of brain infiltration by a fraction of the transplanted hematopoietic progenitors, independently from the administration of a preparatory regimen and from the presence of a disease state in the brain. However, only the use of a conditioning regimen capable of ablating functionally-defined brain-resident myeloid precursors allowed turnover of microglia with the donor, mediated by local proliferation of early immigrants rather than entrance of mature cells from the circulation. This model has raised issues that will be addressed in the future. In particular, a detailed characterization of bona fide microglia progenitors selectively ablated by the conditioning regimen applied is of great biological relevance, while the identification of the fraction within HSPC capable of short-term brain homing and local proliferation would be important for optimizing transplant approaches for LSDs with neurological involvement.

ABSTRACT N. 22

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**MONTINI EUGENIO**	
Telethon grant N.	**TGTGSKP1**	
Costs incurred €	**345.500**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

INTEGRATION CORE: INTEGRATION SITE ANALYSIS IN A CLINICAL TRIAL OF LENTIVIRAL VECTOR BASED HEMATOPOIETIC STEM CELL GENE THERAPY FOR METACHROMATIC LEUKODYSTROPHY

Biffi Alessandra (1), Calabria Andrea (1), Biasco Luca (1), Cesani Martina (1), Benedicenti Fabrizio (1), Plati Tiziana (1), Leo Simone (2), Zanetti Gianluigi (2), Aiuti Alessandro (1), von Kalle Christof (3), Schmidt Manfred (3), Sessa Maria (4), Naldini Luigi (1), Montini Eugenio (1)

(1) San Raffaele Telethon Institute for Gene Therapy, via Olgettina 58, Milan, Italy, Tel. +39.02.26433869, E-mail: montini.eugenio@hsr.it
(2) Center for Advanced Studies, Research, and Development in Sardinia, Pula, Italy
(3) National Center for Tumor Diseases, Heidelberg, Germany
(4) Pediatric Immunohematology and Bone Marrow Transplant Unit, Pediatric and Maternal Department, San Raffaele Scientific Institute, Milan, Italy

A self-inactivating lentiviral vector (LV) has been used in an ongoing hematopoietic stem cell (HSC)-based clinical trial for metachromatic leukodystrophy (MLD) in Milan. In 5 treated patients we obtained multi-lineage hematopoietic reconstitution with up to 80% vector marking. To assess the safety and efficacy of gene transfer as well as the dynamics of hematopoietic reconstitution we studied the integration site profile LAM PCR and high-throughput 454-pyrosequencing in different cell lineages and time points after transplantation (1, 3, 6, 9 and 12 months) in the first 3 MLD patients.
Overall, >32000 unique integrations from the 3 analyzed patients were obtained. The LV integration profile in this trial is highly similar to the one described in the LV-based adrenoleukodystrophy clinical trial. Common Insertion Sites (CIS) were clustered at specific mega-base wide chromosomal regions and were enriched in chromatin remodeling and HLA genes, suggesting that these CIS may be originating by a viral integration bias rather than genetic selection.
Analysis of sequencing reads as a surrogate of the abundance of specific cell clones shows a variable contribution of multiple clones without evidence of clonal dominance. Importantly, tracking of vector integrations across linages and time allow us to confirm HSC marking, the HSC contribution levels within committed hematopoietic lineages and to characterize distinctive dynamics hematopoietic reconstitution between myeloid and lymphoid lineages after transplantation.
Overall, our data show that in in MLD treated patients unprecedented high vector marking levels were achieved without overt evidence of genotoxicity.

ABSTRACT N. 23

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**GRITTI ANGELA**	
Telethon grant N.	**TGT11B02**	
Total budget approved €	**1.094.900**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

CNS-DIRECTED GENE/CELL THERAPY OF LSDs

Ricca Alessandra (1,2), Lattanzi Annalisa (1,2), Cavazzin Chiara (1), Meneghini Vasco (1), Frati Giacomo (1), Corno Daniela (1), Martino Sabata (2), Ungari Daniela (1), Biffi Alessandra (1), Naldini Luigi (1), Gritti Angela (1)

(1) San Raffaele Scientific Institute, San Raffaele Telethon Institute for Gene Therapy (TIGET), Via Olgettina 58, Milan, Italy; Tel +39 0226434623; Fax +39 0226434668; E-mail: gritti.angela@hsr.it
(2) Dept. of Exp. Medicine and Biochemical Sciences, Univ. of Perugia, Italy

Metachromatic and Globoid cell Leukodystrophy (MLD, GLD) are neurodegenerative Lysosomal Storage Disorders (LSD) caused by genetic defects in the activity of arylsulfatase A (ARSA) and galactosylceramidase (GALC), respectively, key enzymes in the catabolism of myelin-enriched sphingolipids. MLD and GLD affect the CNS,

PNS, and the visceral organs. Important issues to be considered in the attempt to develop effective therapies are: i) the multiple pathophysiological processes that combine to create symptoms likely preclude relying on a single therapeutic strategy to restore function; ii) treatment of the CNS pathology remains a challenge; iii) the pathophysiology of these diseases is still largely unknown. Gene therapy (GT) can provide a permanent source of the deficient enzyme in the CNS. This might be achieved either by direct intracerebral injection of vectors or by transplantation of gene-corrected cells. In this latter approach, the biology of neural stem cells (NSC) and hematopoietic stem cells (HSC) might be exploited. Results obtained in the past funding period gave proof of principle for feasibility and efficacy of intracerebral GT and NSC GT as potential approaches for the treatment of CNS pathology in GLD and MLD (Lattanzi et al., HMG 2010; Neri et al., Stem Cells 2011). In this project we focus on the hypothesis that a combinatorial strategy based on direct intracerebral GT or NSC GT coupled to HSC GT (Gentner et al., Sci Transl Med 2010), designed to target different sites of pathology within a suitable window-of-opportunity, may represent a possible solution to these diseases. We have optimized combined protocols and we are assessing their efficacy in appropriate murine models. In parallel, since the progression of these approaches toward human clinical trials requires evaluation in large animals models, we are testing the feasibility and tolerability of the intracerebral GT platform in non-human primates. This will provide us with critical data on the biodistribution of vector and transgenes, also addressing potential safety concerns. A better knowledge of the pathogenesis of MLD and GLD is instrumental to develop effective therapies. We have recently showed impaired organization and function of neurogenic niches in Twitcher mice (GLD model), suggesting a role of GALC in regulating neurogenesis and gliogenesis during CNS development (Santambrogio et al., HMG 2012). In order to model in vitro the onset and progression of CNS pathology we have now established patient-specific induced pluripotent stem cells (iPSCs) by somatic cellular reprogramming, generating iPSC-derived neurons and glia. On these cultures we are assessing functional features, biochemical, molecular and epigenetic signature, trying to model the degenerative mechanisms, to test the efficacy of gene correction in reverting the disease phenotype and, ultimately, to test safety and efficacy of iPSC-derived neural cells in the appropriate murine models.

ABSTRACT N. 24

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**AIUTI ALESSANDRO**	
Telethon grant N.	**TGT11C01**	
Total budget approved €	**739.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

TRACKING AND MODELING OF HSC CLONAL DYNAMICS BY VECTOR MARKING

Luca Biasco (1), Cristina Baricordi (1), Serena Scala (1,2), Francesca Dionisio (1), Andrea Calabria (1), Samantha Scaramuzza (1), Nicoletta Cieri (3), Danilo Pellin (4), Clelia Di Serio (2,4), Cynthia Bartholomae (5), Chiara Bonini (3), Christof Von Kalle (5), Manfred Schmidt (5), Eugenio Montini (1), Luigi Naldini (1,2), Alessandro Aiuti (1,6)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132, Milano, Italy, Tel. +390226434671, Fax +390226434668, E-mail: alessandro.aiuti@hsr.it
(2) Università Vita-Salute San Raffaele, Milano, Italy
(3) San Raffaele Scientific Institute, HSR, Italy
(4) CUSSB, Università Vita-Salute, Milan Italy
(5) National Center for Tumor Diseases (NCT-DKFZ), Heidelberg, Germany
(6) University of Rome "Tor Vergata", Rome, Italy

The study of hematopoiesis and hematopoietic stem cells (HSC) dynamics in humans relies on data derived from in vitro experiments or animal models, but direct evidences on clonal tracking in humans are currently missing. Upon retroviral gene transfer, transduced cells are univocally marked by vector integration sites (IS). Profiling of IS derived from Wiskott–Aldrich syndrome (WAS) and ADA (adenosine deaminase) deficient-SCID gene therapy (GT) patients treated at HSR-TIGET allow us to study HSC in the early and late timepoints after transplant, respectively. From high-throughput analyses we collected 23.156 IS in 3 WAS patients up to 1 year and 3.054 IS in 4 ADA-SCID patients from 4 to 6 years after GT. In the early phases after transplant in WAS patients we detected highly polyclonal haematopoiesis resulting from subsequent distinct hematopoietic clonal output of transduced HSPC. Remarkably, the diversity of lineage repertoires increased between 6 and 12 months in the absence of sustained clonal expansions or skewing towards integrations near protoncogenes. Studying long term HSC activity by a tri-factorial analysis of insertional variables on ADA-SCID patients, we found that BM cells and PB granulocytes cluster differently from PB lymphoid lineages due to the effects of selective advantages of gene corrected T and B cells. Bayesian nets based approaches allowed us to reconstruct the basic shape of the hematopoietic tree underlining the biological consistency of insertion site similarities among lineages. We identified "core integrants" shared between CD34+ cells and both lymphoid and myeloid cells, tagging long-term repopulating stem cells. By specific PCRs (on integrants inside MLLT3 and downstream the LRRC30 genes) we were able to track the dynamics and output activity of two of these HSC clones over a period of 5 years, showing that cytokine exposed non-dormant HSC can preserve their multilineage potential years after infusion. Another research line is focused on studying patients who received infusions of transduced lymphocytes (PBL-GT) and aimed at tracking single T-cell clones and investigating the survival potential and hierarchical relationships of naive and memory subpopulations directly in vivo in humans. We found that transduced T cells with an apparent naïve phenotype (CD45RA+/62L+) share the highest percentage of insertions (41.2%) with other T subpopulations while still surviving in vivo 10 years after infusion. The vast majority of phenotypically naive cells (92.5%) in PBL-GT patients are indeed the recently defined CD95+ T memory stem cells (TSCM) carrying long-term survival capacity coupled with naive-like plasticity. Our preliminary data show that TSCM could be generated in vitro following PBL-GT transduction protocol. In summary, IS analyses are providing novel information on hematopoietic dynamics with a potential impact on GT and broader therapeutic approaches for hematopoietic diseases.

ABSTRACT N. 25

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**FERRARI GIULIANA**	
Telethon grant N.	**TGT11C02**	
Total budget approved €	**736.200**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

PRELIMINARY RESULTS OF SAFETY AND EFFICACY OF CD34+ CELLS MOBILIZATION AFTER PLERIXAFOR ADMINISTRATION AS SINGLE AGENT IN ADULT THALASSEMIC PATIENTS: TOWARDS THE ASSESSMENT OF THE SUITABLE HEMATOPOIETIC STEM CELL SOURCE FOR GENE THERAPY OF BETA-THALASSEMIA

Lidonnici Maria Rosa (1,5), Aprile Annamaria (1), Frittoli Marta (1,5), Mandelli Giacomo (1), Gentner Bernhard (1,2,5), Bellio Laura (3), Cassinerio Elena (4), Zanaboni Laura (4), Rossini Silvano (3), Cappellini Maria Domenica (4), Ciceri Fabio (2), Marktel Sarah (2), Ferrari Giuliana (1,5)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milan, Italy, Tel. 0226434705, Fax 0226434668, E-mail: ferrari.giuliana@hsr.it
(2) Hematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan, Italy
(3) Immunohematology and Transfusion Medicine Unit, San Raffaele Scientific Institute, Milan, Italy
(4) Università di Milano, Ca Granda Foundation IRCCS, Milan, Italy
(5) Università Vita-Salute San Raffaele, Milan, Italy

Gene therapy of inherited blood diseases requires transplantation and engraftment of a high number of autologous genetically engineered hematopoietic stem cells (HSCs). In order to obtain the correction of the disease, a favorable balance between the extent of conditioning of the host bone marrow (BM) and the adequate dose of transplanted, transduced HSCs needs to be achieved. In the clinical application of gene therapy for adult thalassemic patients, the issue of HSC procurement is crucial as the minimal target dose poses a challenge for the use of steady state BM. Peripheral blood mobilized CD34+ cells represent a valid alternative, but intrinsic characteristics of thalassemic patients (i.e. splenomegaly and thrombophilia) dictate caution in the choice of HSC source, especially for the use of G-CSF as mobilizing agent.
A clinical protocol exploring the use of Plerixafor (AMD3100) as single agent was submitted and approved (acronym AMD-THAL, EudraCT 2011-000973-30). Plerixafor selectively and reversibly antagonizes the binding of stromal cell-derived factor-1 to its receptor

CXC motif receptor-4 with subsequent egress of HSC to the peripheral blood. Aims of the AMD-THAL phase I-II clinical trial were to explore the ability of Plerixafor in inducing safe and effective stem cells mobilization in adult patients affected by beta-thalassemia, to characterize stem/progenitor cells mobilized from the BM and peripheral blood of treated subjects and to achieve gene transfer efficiency of mobilized CD34+ cells at a level comparable to that obtained using steady state BM.
Four subjects affected by transfusion dependent beta-thalassemia were enrolled and treated. Plerixafor was administrated subcutaneously as single agent and at the single dose of 0.24 mg/kg. Only one patient received a second dose at 0.40 mg/kg 24 hrs after the first dose and underwent a second leukoapheretic procedure. Three out of four patients achieved the minimal target cell dose (2 x 106 cells/kg), but not the optimal harvest of 5 x 106 CD34+ cells/kg. No severe adverse events occurred. In addition, steady state and Plerixafor primed BM aspirates were performed to analyze any modification in CD34+ concentration in the BM following Plerixafor administration. Plerixafor administration resulted in enrichment of CD34+ cells concentration in the BM.
Purified CD34+ cells from leukoaphereses were analyzed for their biological and functional properties, subpopulations composition and expression profile. In vivo reconstitution potential and lymphomyeloid differentiation of human CD34+ cells are tested following transplantation in NSG mice. We also compared Plerixafor-mobilized peripheral blood cells with BM CD34+ cells pre- and post-Plerixafor treatment, derived from the same patient. Cells were transduced with a lentiviral vector carrying human beta-globin gene (GLOBE) to assess gene transfer efficiency. The results indicate that cells mobilized by Plerixafor have a primitive phenotype with a high reconstitution potential and are efficiently transduced, thus being a suitable source of target cells for gene therapy.

ABSTRACT N. 26

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**FERRARI GIULIANA**	
Telethon grant N.	**TGTGSK04**	
Costs incurred €	**327.300**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

PRECLINICAL SAFETY EVALUATION OF GENE THERAPY MEDICINAL PRODUCTS: TUMORIGENICITY AND TOXICOLOGY STUDY TO SUPPORT A BETA-THALASSEMIA GENE THERAPY CLINICAL TRIAL

Salvatori Francesca (1), Tiboni Francesca (1), Mandelli Giacomo (1), Aprile Annamaria (1), Lidonnici Maria Rosa (1,3), Sanvito Francesca (2), Cristofori Patrizia (4), Palini Alessio (2), Oriani Enzo (2), Naldini Luigi (1,3), Ferrari Giuliana (1,3)

(1) (1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) - Via Olgettina 58, 20132, Milano, Italy, Tel 02 26434705, Fax 02 26434668 E-mail: giuliana.ferrari@hsr.it
(2) San Raffaele Scientific Institute, Milan
(3) Vita-Salute San Raffaele University, Milan, Italy
(4) Glaxo Smith Kline, R&D, Ware, UK

A few pioneering gene therapy clinical trials have demonstrated the therapeutic potential of gene-modified hematopoietic cells to correct genetic disorders. Rigorous preclinical studies in the most appropriate models are of paramount importance to assess the risk/benefit ratio and fulfill the requirements for future market registration. Evaluating biosafety according to regulatory guidelines for gene therapy medicinal products (GTMP), utilizing GLPs (good laboratory practices), will provide results of utmost scientific and regulatory significance. Therefore, we have started developing a GLP Test Facility within the academic HSR-TIGET and a preclinical study for beta-thalassemia gene therapy.
The study will assess the potential tumorigenicity and toxicity induced by transplantation of murine hematopoietic stem/progenitor cells (HSPCs) transduced with a lentiviral vector (LV) containing the human beta-globin gene (GLOBE) in the murine disease model of thalassemia.
The study was designed by evaluating the choice of the animal model, size of experimental groups, selection of dose/route of administration, study duration, sampling type and time points and analysis at termination. The selected cell dose was chosen as multiple of that expected in humans and the transduction protocol was designed to achieve a vector copy number (VCN)/cell equal or superior to that expected in human cells.
The transduced cells (Test Item) were obtained by gene transfer into HSPCs, purified as Lin^- cells from mutant thalassemic (*th3/+*) mice and were transplanted in recipient *th3/+* mice pre-treated with Busulfan, which represent the Test System (n=30). Mock-transduced cells (Control Item), consisting of Lin^- cells cultured as the Test Item in absence of the LV, were transplanted in recipient mice. In parallel, control untreated age-matched mice are monitored. The study duration will be 12 months. Test and Control Items were produced in 3 independent batches, each characterized by evaluating cell viability, clonogenic capacity (CFU) and mean VCN/cell on CFU. The first time-point analysis on treated and mock-treated mice was performed at 16±2 weeks after transplantation, consisting of evaluation of VCN in peripheral blood, transgene expression by haemoglobin (Hb) concentration and detection of mouse-human chimeric Hb.
Test Item cell viability was 72±6.8%, CFU number was $3.2x\pm0.16x10^4$ CFU/10^6 cells, VCN/cell on CFU was 8.5±1.8, and transduction efficiency was 100%. Control Item cell viability was 71±6.8%, CFU number was $2.5x\pm 0.17x10^4$ CFU/10^6 cells.
At 16 weeks, VCN/cell in blood was 9.4±2.3 (male), 8.3±3.1 (female) and all the haematological parameters show values indicating disease correction. So far, no abnormal values were recorded and all treated animals are alive and well.

ABSTRACT N. 27

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**MONTINI EUGENIO**	
Telethon grant N.	**TGT11D01**	
Total budget approved €	**731.800**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

IMPROVING SAFETY OF LENTIVIRAL GENE TRANSFER: IN VIVO MOUSE MODELS FOR VECTOR GENOTOXICITY TESTING AND LENTIVIRAL VECTOR-BASED CANCER GENE DISCOVERY

Cesana Daniela (1), Ranzani Marco (1), Volpin Monica (1), Bartholomä Cynthia (2), Benedicenti Fabrizio (1), Merella Stefania (1), Sergi Sergi Lucia (1), Sanvito Francesca (3), Brombin Chiara (4), Nonis Alessandro (4), Di Serio Clelia (4), Doglioni Claudio (3), Von Kalle Christof (2), Schmidt Manfred (2), Naldini Luigi (1), Montini Eugenio (1)

(1) San Raffaele Telethon Institute for Gene Therapy - via Olgettina 58, Milan, Italy, Tel. +390226433869, Fax +390226434621, E-mail: montini.eugenio@hsr.it
(2) National Center for Tumor Diseases, Heidelberg, Germany
(3) San Raffaele Department of Pathology, Milan, Italy
(4) Centro Universitario di Statistica per le Scienze Biomediche, Milan, Italy

We developed a novel in-vivo vector integration genotoxicity assay based on systemic vector injection into newborn tumor-prone Cdkn2a-/- and heterozygous Cdkn2a+/- mice. Treatment with an LV with self-inactivating (SIN) LTR, harboring the Spleen-Focus-Forming-Virus (SF) enhancer/promoter in internal position and driving the GFP expression, caused a significant acceleration in hematopoietic tumor onset with respect to controls in both mouse models. The treatment with a vector devoid of the Open-Reading-Frames (ORF) downstream the SF promoter induced a more dramatic acceleration of tumor onset even in a SIN LTR configuration. By analyzing >7500 tumors-derived LV integrations, we identified 72 Common Insertion Sites (CIS), indicating that tumor onset acceleration was caused by insertional mutagenesis. Interestingly, we found that the ORF-less LV induced oncogenesis mainly by the activation of Braf by a read-through/splicing-capture mechanism. On the other hand the LV containing the ORF, being unable to activate Braf, activated/disrupted other oncogenes or tumor-suppressor genes through enhancer-mediated and/or aberrant splicing mechanisms. Braf activation appears to be a highly dominant and powerful oncogene in our mouse models while the activation of other oncogenes induces a delayed tumor onset. The expression levels of the human orthologs of the CIS genes found in our study in Acute Myeloid Leukemia patients (n=229, 2 independent cohorts) were correlated by multivariate analysis to their survival. Interestingly, the expression of 2 CIS genes significantly impacted the patients' survival. Hence, our approach can be used to study the genotoxicity of integrative vectors and to discover novel clinically relevant cancer genes.

ABSTRACT N. 28

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**NALDINI LUIGI**	
Telethon grant N.	**TGTGSKP2**	
Costs incurred €	**191.700**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

A microRNA DIMMER SWITCH FOR HEMATOPOIETIC STEM CELL GENE THERAPY

Gentner Bernhard (1,2), Zonari Erika (1), Giustacchini Alice (1,2), Boccalatte Francesco (1,2), Plati Tiziana (1), Biffi Alessandra (1,2), Aiuti Alessandro (1,2), Naldini Luigi (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy, Via Olgettina 58, 20132 Milano, Tel 02.26434681, Fax 02.26434621/4668, E-mail: naldini.luigi@hsr.it
(2) Vita Salute San Raffaele University

Hematopoietic stem cell (HSC) gene therapy has made significant progress over the last years and currently offers a therapeutic option for several genetic diseases which is potentially less risky and more effective than allogeneic stem cell transplantation. We have harnessed microRNA activity to negatively regulate transgene expression in distinct hematopoietic subpopulations to achieve – in combination with appropriate transcriptional control elements, lineage-restricted expression profiles with unprecedented specificity. Exploiting the HSC and progenitor cell-specific expression profile of miR-126 and miR-130a, we have constructed "HSC-off" vectors lacking expression in hematopoietic stem cells as opposed to differentiated cells, thus allowing the delivery of a transgene into HSC without altering their proteome, while benefitting from sustained multi-lineage expression in their progeny. We have defined the optimal combination of miRNA target sequences to yield regulation indexes of 10-20 fold, and minimizing the chance of saturating the endogenous activity of miR-126. We have also performed in-depth biological studies on the function of miR-126 in normal hematopoiesis (Lechman et al, Cell Stem Cell 2012) and found that miR-126 knockdown resulted in HSC expansion in the absence of exhaustion or oncogenesis, a benign and potentially desirable phenotype further mitigating concerns on miR-126 saturation.
We have also characterized a novel myeloid specific promoter (Barde et al, Gene Ther 2011) in healthy donor bone marrow cells. This promoter shows a more than 30 fold induction between HSC and differentiated myeloid cells, with expression levels surpassing those of the ubiquitous PGK promoter in the latter compartment. In combination with miRNA-mediated detargeting from HSC, we could achieve a more than 300 fold induction of transgene expression during myeloid differentiation, making this vector design a promising candidate for clinical development of gene therapy for chronic granulomatous disease and globoid leukodystrophy.

ABSTRACT N. 29

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**NALDINI LUIGI**	
Telethon grant N.	**TGT11D02**	
Total budget approved €	**918.400**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

EXPLOITING ARTIFICIAL NUCLEASES FOR TARGETED GENE CORRECTION OF SCID-X1 PATIENT-DERIVED HEMATOPOIETIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

Lombardo Angelo (1), Genovese Pietro (1,2), Firrito Claudia (1), Di Tomaso Tiziano (1), Escobar Giulia (1), Schiroli Giulia (1,2), Gennery Andrew R. (3), Sergi Sergi Lucia (1), Gregory Philip D. (4), Holmes Michael C. (4), Naldini Luigi (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy, San Raffaele Hospital, Via Olgettina 58, Milan, Italy, Tel. 0226434681, Fax 0226434668, E-mail: naldini.luigi@hsr.it
(2) Vita-Salute San Raffaele University, Milan, Italy
(3) Newcastle Upon Tyne Hospital, Newcastle Upon Tyne, United Kingdom
(4) Sangamo BioSciences Inc., Richmond, CA, United States

The development of artificial nucleases has brought the possibility of targeted integration and gene correction within the reach of gene therapy. In this project, we exploited engineered Zinc Finger Nucleases (ZFNs) to induce homology-driven transgene insertion into a predetermined genomic site and used this strategy to correct mutations in Hematopoietic Stem/Progenitor Cells (HSPC) and induced Pluripotent Stem Cells (iPSC) from X-linked Severe Combined Immunodeficiency (SCID-X1) patients. This disease, which is caused by mutations in the IL2RG gene, is an ideal candidate to test efficacy and safety of this novel approach, as HSPC-based gene therapy trials performed with randomly integrating vectors showed clinical benefits but also a high rate of leukemia due to insertional mutagenesis and uncontrolled transgene expression. To achieve ZFN-mediated targeted insertion in HSPC, we developed a combined gene delivery protocol based on integrase-defective Lentiviral Vectors (LV) and mRNA nucleofection that allows providing the donor template DNA for homologous recombination while transiently expressing ZFNs. By using this protocol we can either target a transgene expression cassette into the AAVS1 "safe harbor" locus or insert a functional corrective IL2RG cDNA downstream of its endogenous promoter with unprecedented efficiency and high specificity in HSPC. The treated HSPC generated both erythroid and myeloid colonies in vitro of gene targeted cells and, upon transplantation into NSG mice, reproducibly gave rise to both myeloid and lymphoid lineages. Importantly, gene-targeted cells were also found in the mice within the primitive human hematopoietic compartment, thus providing indication of targeted integration into long-term repopulating stem cells. In parallel, we have explored the use of patient-derived iPSC as a potentially unlimited source of gene-corrected HSPC. We have established a strategy that allows correction of the IL2RG gene and safe reprogramming of these cells. Using the ZFNs technology we inserted the corrective IL2RG cDNA in fibroblasts from SCID-X1 patients with high efficiency. To select the gene-corrected fibroblasts, which do not express IL2RG, we included downstream of the corrective cDNA a loxP-flanked selector cassette, and efficiently reprogrammed the selected cells to iPSC by using a single-copy, Cre-excisable LV expressing the reprogramming factors. Transient Cre delivery resulted in near complete excision of the reprogramming LV and the selector cassette from the iPSC genome, demonstrating the feasibility of generating gene-corrected and reprogramming factor-free iPSC from SCID-X1 patients, which are currently tested for differentiation into HSPC. Overall, these studies provide proof-of-principle of specific ZFN-mediated gene targeting and correction in patient-derived HSPC and iPSC, and open the way to develop a more precise and safe gene therapy strategy for SCID-X1 and, conceivably, several other diseases.

ABSTRACT N. 30

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**NALDINI LUIGI**	
Telethon grant N.	**TGT11D03**	
Total budget approved €	**1.100.300**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

EFFECTIVE AND SAFE LIVER GENE THERAPY OF HEMOPHILIA B IN MICE AND DOGS BY LENTIVIRAL VECTORS AND HYPER-FUNCTIONAL TRANSGENES

Cantore Alessio (1,2), Ranzani Marco (1,2), Della Valle Patrizia (3), Bartolaccini Sara (1), Sergi Sergi Lucia (1), VandenDriessche Thierry (4), Chuah Marinee (4), Bellinger Dwight (5), D'Angelo Armando (3), Nichols Timothy (5), Montini Eugenio (1), Naldini Luigi (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy, San Raffaele Hospital, Via Olgettina 58, Milan, Italy, Tel. 0226434681, Fax 0226434668, E-mail: naldini.luigi@hsr.it
(2) "Vita Salute San Raffaele" University, Milan, Italy
(3) Coagulation Service, San Raffaele Scientific Institute, Milan, Italy
(4) The Free University of Brussels, Brussels, Belgium
(5) University of North Carolina, Chapel Hill, USA

Lentiviral vectors (LVs) are attractive vehicles for liver gene therapy, given a sizeable transgene capacity and their ability to integrate in the target cell genome. Moreover, most human subjects have no pre-existing humoral or cellular immunity against vector components. We have developed microRNA 142-regulated LVs that stringently target transgene expression to hepatocytes and induce immune tolerance to transgene proteins. We have shown that these LVs can fully and stably correct the disease phenotype in hemophilia B (hemoB) mice. We are currently evaluating this strategy in a large animal model of the disease. Our results show long-term canine Factor IX (FIX) expression up to 1% of normal levels and clinical improvement (almost complete prevention of spontaneous bleedings) in two hemoB dogs, with up to 3.5 years follow up. Whereas these data indicate efficacy of gene therapy, in order to further improve FIX activity reconstitution, we generated hyper-

functional FIX transgenes carrying a R338L amino acid substitution, previously associated with clotting hyperactivity and thrombophilia in humans. The hyper-functional FIX transgene and its codon-optimized (co) version increased FIX activity reconstituted in the plasma of hemoB mice, without detectable adverse effects, allowing correction of the disease phenotype at low vector doses, thus improving the efficacy, feasibility and safety of our gene therapy strategy. Treatment of a hemoB dog with this hyper-functional FIX transgene is underway.
Whereas we did not observe any adverse effect in mice treated with liver gene transfer, to stringently assess the oncogenic risk associated with vector integration we developed sensitive mouse models of genotoxicity in tumor-prone Cdkn2a-/- or wild type (WT) mice. As positive control of insertional mutagenesis, we engineered LV to carry hepatocyte-specific enhancer/promoters in the Long Terminal Repeats (LV.ET.LTR) and induced hepatocellular carcinoma (HCC) in 30% of Cdkn2a-/- mice and 75% of WT mice in combination with CCl4 treatment. Systemic administration of the therapeutic FIX LV did not induce HCCs in tumor prone Cdkn2a-/- (n=39) or in WT mice treated with CCl4 (n=23). We then retrieved 9,215 unique insertion sites from the treated mice. Whereas integrations of the genotoxic vector were enriched at previously validated HCC oncogenes, indicating positive selection of cancer-causing events, the therapeutic LV did not show an integration bias for these genes. Moreover, we did not detect any evidence of selection for the therapeutic LV insertions. These data indicate a low risk of insertional oncogenesis by LV liver gene therapy and position our platform for further development and clinical translation.

ABSTRACT N. 31

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**RONCAROLO MARIA GRAZIA**	
Telethon grant N.	**TGT11E01**	
Total budget approved €	**954.300**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

LIVER-DIRECTED GENE TRANSFER FOR THE INDUCTION OF ANTIGEN-SPECIFIC TOLERANCE: MECHANISM AND POTENTIAL THERAPEUTIC APPLICATIONS

Annoni Andrea (1), Goudy Kevin (1), Cantore Alessio (1), Akbarpour Mahzad (1), Naldini Luigi (1,2), Roncarolo Maria Grazia (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milano, Italy, Tel. +39.02.26434875 Fax +39.02.26434668;E-mail: roncarolo.mariagrazia@hsr.it
(2) Vita-Salute San Raffaele University, Milano, Italy

We have developed an efficient lentiviral vector (LV) platform for liver gene transfer, which combines two layers of transgene regulation: transcriptional (hepatocyte-specific promoter, (ET)) and post-transcriptional (miR-142-target sequences (142T)). We previously showed that the LV.ET.142T platform, even in its integrase-defective form, induces a robust state of active tolerance towards the encoded antigen (Ag) which is mediated by de novo induction of Ag-specific, Foxp3+ T regulatory cells (iTreg) (Annoni et al. Blood 2009) (Matrai et al. Hepatology 2011).
Based on these results we sought to investigate the mechanisms of iTregs induction in the liver after LV.ET.142T treatment. We set up an in vivo model to study Ag-specific iTreg induction in the first two weeks after gene transfer in mice treated with LV.ET.142T, or LV.PGK (immunogenic control). We found that TGF-beta is up-regulated in LV.ET.142T mice in the second week after treatment, and that this upregulation correlates with the Ag-specific iTreg differentiation. Blocking TGF-beta by a neutralizing antibody reduced induction of Foxp3+ cells to levels detected in LV.PGK mice.
We also investigated which subset of liver cells plays a role in inducing iTreg by providing TGF-beta and/or Ag presentation. Gene expression studies on cells isolated seven days post LV gene transfer revealed a 10-fold increase TGF-beta levels in liver parenchymal cells, suggesting that hepatocytes are the key TGF-beta producing cells upon LV.ET.142T treatment. In vitro stimulation of OTII CD4+ T cells (TCR transgenic OVA 323-339-specific) did not reveal a preferential role of any non-parenchymal cell subset, although all of them possess Ag presenting capacity.
To explore the tolerogenic and therapeutic potential of the LV.ET.142T platform, we studied the capacity of LV.ET.142T vector to resolve ongoing detrimental immune reactions in two preclinical models. To test the ability of LV.ET.142T to overcome pre-existing neutralizing antibodies (nAbs), hemophilia B mice (F.IX-/-) were challenged with F.IX to induce nAbs. After LV.ET.F.IX.142T treatment anti-F.IX neutralizing response was progressively resolved and F.IX rose to 20% of normal levels. To evaluate the ability of LV.ET.142T to resolve ongoing autoimmunity, NOD mice were treated with LV.ET.142T encoding insulin B (9-23) fragment at 10 weeks of age and resulted to be protected from type-1 diabetes.
These results indicate that liver tolerogenic properties can be exploited by the use of LV.ET.142T platform encoding for model Ag, which induces Ag specific tolerance. Findings from these studies prove the versatility of the LV.ET.142T platform to correct monogenic diseases and to identify novel therapeutic approaches to induce tolerance in immune mediated disorders.

ABSTRACT N. 32

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**GREGORI SILVIA**	
Telethon grant N.	**TGT11E02**	
Total budget approved €	**710.600**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2011**

CELL THERAPY STRATEGIES FOR TOLERANCE INDUCTION IN HUMANS: IL-10 ENGINEERED T CELLS

Andolfi Grazia (1), Locarafo Grazia (1), Russo Fabio (1), Camisa Barbara (2), Attilio Bondanza (2), Maria Grazia Roncarolo (1,2), Silvia Gregori (1)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. Tel 02.26434669, Fax 02.26434668, E-mail: gregori.silvia@hsr.it
(2) Experimental Hematology Unit, PIBIC, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
(3) Università Vita-Salute San Raffaele University, Milan, Italy

The induction of transgene(Tg)-specific tolerance is a fundamental task for the clinical development of successful gene transfer protocols, otherwise hampered by the immune-mediated clearance of engineered cells. Regulatory T cells (Tregs) are essential for inducing and maintaining tolerance, thereby they represent a good candidate to inhibit T-cell mediated responses, including those associated with gene transfer. In humans two main subtypes of CD4+ Tregs have been described: FOXP3+ Tregs and inducible IL-10-producing Tr1 cells. Tr1 cells control immune responses via IL-10 and the killing of myeloid cells through the release of Granzyme B. Tr1 cells can be induced in vitro using a new subset of human tolerogenic DC, termed DC-10 (Gregori S, et al. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. Front Immunol. 2012;3:30. Epub 2012 Feb 29).
Although DC-10 are efficient in promoting Tr1 cells, the resulting population includes a significant fraction of contaminating non-Tr1 cells, representing a major drawback for clinical application of Tr1 cells. To overcome this limitation, we developed a bidirectional lentiviral vector (LV) encoding for human IL-10 and the marker gene protein (DNGFR), which are independently co-expressed. Enforced LV-mediated IL-10 expression confers to human CD4+ T cells the ability to secrete high levels of IL-10 and concomitant low levels of IL-4, and markers associated with IL-10. $CD4^{IL-10}$ T cells are anergic and display a potent ability to suppress T cell responses in the IL-10, and transforming growth factor (TGF)-β dependent fashion and in a cell-to-cell contact independent manner in vitro. Using a clinical relevant humanized mode of xeno graft-versus-host disease (xeno-GvHD) in immune-compromized NSG mice we demonstrated that single injection of $CD4^{IL-10}$ T cells is sufficient to control xeno-GvHD mediated by allogenic CD4+ or peripheral blood mononuclear cells (PBMC) in vivo (Andolfi G, et al. Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells. Mol Ther. 2012 Sep;20(9):1778-90. doi: 10.1038/mt.2012.71. Epub 2012 Jun 12). These results show that constitutive over-expression of IL-10 in human CD4+ T cells leads to a stable cell population that recapitulates the phenotype and function of Tr1 cells. The LV-IL-10 platform has been also applied to generate human allo-specific $CD4^{IL-10}$ T cells. To this end human CD4+ T cells were stimulated with allogeneic in vitro differentiated DC and activated T cells have been isolated based on the co-expression of early T cell activation markers. FACS-sorted T cells were transduced with LV-IL-10. Resulting allo-specific $CD4^{IL-10}$ T cells, upon allo-specific stimulation, secrete IL-10, are anergic, and suppress allo-specific T cell responses in vitro.
In conclusion we developed a suitable method to generate allo-specific IL-10-engineered T cells. These results represent the first step for the development of antigen-specific IL-10-producing T cells and

will contribute to increase the success of Tr1-based immunotherapy, inducing tolerance to selected antigens, while minimizing general immune suppression.

ABSTRACT N. 33

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**BIFFI ALESSANDRA**	
Telethon grant N.	**TGTGSK05**	
Costs incurred €	**993.000**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

HEMATOPOIETIC STEM CELL GENE THERAPY FOR THE TREATMENT OF TYPE I MUCOPOLYSACCHARIDOSIS

Visigalli Ilaria (1), Delai Stefania (1), Ferro Francesca (1), Cecere Francesca (1), Politi Letterio Salvatore (2), Quattrini Angelo (3), Sanvito Francesca (4), Rovelli Attilio (5), Parini Rossella (5), Boelens Jaap J (6), Di Natale Paola (7), Naldini Luigi (1,8), Biffi Alessandra (1,9,10)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132, Milan, Italy, Tel. 0226434678; Fax 0226434668; E-mail: a.biffi@hsr.it
(2) Neuroradiology Group, Imaging Core, San Raffaele Scientific Institute, Milan, Italy
(3) Experimental Neuropathology Unit, Neuroscience Dept., San Raffaele Scientific Institute, Milan, Italy
(4) Mouse HistoPathology Unit, San Raffaele Scientific Institute, Milan, Italy
(5) Pediatric Blood and Marrow Transplantation Program, UMC Utrecht, Netherland
(6) MBBM Foundation, Pediatric Department, Milano-Bicocca University at San Gerardo Hospital, Monza, Italy
(7) Department of Biochemistry and Medical Biotechnology, University of Naples Federico II, Naples, Italy
(8) Vita Salute San Raffaele University, Milano, Italy
(9) HSR-TIGET Pediatric Clinical Research Unit, San Raffaele Scientific Institute, Milan, Italy
(10) Pediatric Immunohematology, San Raffaele Scientific Institute, Milan, Italy

Type 1 Mucopolysaccharidosis (MPS I) is an inherited lysosomal storage disorder caused by the deficiency of a-L-iduronidase (IDUA), and is characterized by accumulation of mucopolysaccharides within the lysosomes in different tissues. This leads to multisystemic impairment, with visceromegaly, organ insufficiency, skeletal abnormalities, dysmorphism and mental delay. According to the severity of symptoms, three disease variants are distinguished: the mild Schieie syndrome, the severe Hurler syndrome (MPS I-H, characterized by central nervous system involvement) and the intermediate Hurler-Scheie form. Available treatments such as enzyme replacement therapy, restricted to Schieie/Hurler-Scheie forms, and hematopoietic stem cell transplantation (HSCT), used in MPS I-H, are poorly effective on skeletal and brain disease manifestations. In order to improve the therapeutic efficacy of HSCT, we developed a gene therapy (GT) strategy based on lentiviral vector (LV) use for HSC transduction with the goal of inducing supra-physiological enzyme production by HSCs and in their tissue infiltrating progeny. When tested in the MPS I murine model, HSCGT showed a higher efficacy with respect to normal donors' HSCT in preventing disease onset and/or in correcting neurological and skeletal defects. Based on these results, a clinical development plan for moving this approach to clinical testing has started. To this goal, safety studies started as well, focused on the evaluation of the toxic and tumorigenic potential, and on the biodistribution properties of HSCs transduced with a clinical-grade LV encoding for IDUA. These studies are being conducted in Good Laboratory Practice in order to guarantee the quality and validity of the data. For studying the toxic and tumorigenic potential of the IDUA.LV transduced HSCs, murine HSCs (isolated by Lineage negative (Lin-) selection) are transplanted in MPS I mice since: i) the use of human HSCs from MPS I patients would require high amounts of harvested bone marrow, which is unethical and unfeasible to obtain from pediatric patients; ii) Lin- cells can be considered equivalent to the human CD34+ progenitor cells used in the clinical trial; iii) the toxicology study aims at applying the most appropriate and sensitive model to evaluate any possible risk of tumorigenicity in a preclinical context - humanized chimeric mouse models, due to intrinsic limitations of the model, do not allow such a long-term evaluation, which is instead feasible in the mouse/mouse transplant setting. Concomitantly, for the biodistribution study, experiments to confirm feasibility and safety of the protocol currently employed in the MLD clinical trial for MPS I patients' HSC transduction are on going on HSCs from normal donors with a clinical-grade IDUA.LV. Moreover, studies to compare Busulfan conditioning with total body irradiation in the immunodeficient murine model selected for the biodistribution study are being performed.

ABSTRACT N. 34

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**AIUTI ALESSANDRO**	
Telethon grant N.	**TGTGSK06**	
Costs incurred €	**166.000**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

GENE THERAPY FOR CHRONIC GRANULOMATOUS DISEASE USING REGULATED LENTIVIRAL VECTORS

Chiriaco Maria (2), Farinelli Giada (2), Capo Valentina (2), Scaramuzza Samantha (1), Sergi Sergi Lucia (1), Grez Manuel (3), Trono Didier (4), Finocchi Andrea (1), Rossi Paolo (2), Naldini Luigi (1,5), Gentner Bernhard (1), Aiuti Alessandro (1,2)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, 20132 Milan, Italy, Tel. +390226434671, Fax +390226434668, E-mail: alessandro.aiuti@hsr.it
(2) Department of Pediatrics, Children's Hospital Bambino Gesù and University of Rome Tor Vergata, Rome, Italy
(3) Georg-Speyer Research Institute, Frankfurt, Germany
(4) École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland
(5) Vita-Salute" S.Raffaele University, Milan, Italy

Chronic Granulomatous Disease (CGD) is caused by defective NADPH oxidase function in patients' phagocytes leading to increased susceptibility to fungal and bacterial infections. Gene therapy with hematopoietic stem cells (HSC) may represent a valid alternative for gp91phox deficient patients (X-CGD). Clinical trials with gamma-retroviral vectors have achieved restoration of NADPH oxidase activity but long-term clinical benefit was hampered by low engraftment levels of gene modified cells, transgene silencing, and the occurrence of clonal dominance and myelodysplasia. Since gp91phox is not present in human HSC and its expression was not regulated in these early studies, a negative role of ectopic transgene expression on HSC survival and growth has been also hypothesized. Our goal was to develop a novel gene transfer approach into HSC for safe and effective therapy of X-CGD based on SIN lentiviral vectors (LVV) encoding gp91phox. To reduce the risk of ectopic gp91phox expression in HSC, we introduced four or two miR-126 target sequences (miR-126T) into gp91phox expression cassette (LVV-PGKgp91_126T), resulting in post-transcriptional downregulation of gp91phox expression by miR-126 which is found to high levels in HSC but not in the myeloid progeny (Gentner et al, Gentner B. et al., Sci Transl Med2010). We also generated a LVV that incorporates a myeloid specific promoter (LVV.MSP.gp91) to allow transcriptional regulation of the transgene. The regulated vectors were compared with LV in which gp91phox is constitutively expressed under cellular (LVV-PGKgp91) or viral promoters (LVV-SFFVgp91). Human cell lines, murine Lineage- HSC and human CD34+ cells were transduced and tested for specific expression and function of gp91phox (FACS, DHR, Cytochrome c reduction). All vectors fully restored gp91phox expression and NADPH oxidase activity in human X-CGD myeloid cell lines. In presence of miR-126 expressing LVV, LVV-PGKgp91_126T-transduced gp91phox-deficient cells showed dramatically reduced gp91phox expression. Transduction of X-CGD murine cells and human CD34+ cells resulted in effective restored transgene expression and correction of NADPH activity after in vitro differentiation. After short-term culture, gp91phox was detected ectopically in undifferentiated CD34+ cells transduced with LV.PGK.gp91 driving constitutive transgene expression. In contrast, both LVV-PGKgp91_126T and LV.MSP.gp91 regulation systems showed their efficacy in restricting the expression of gp91phox in HSC from patients and healthy donors, while maintaining it in differentiated myeloid cells. Preliminary results in X-CGD mice showed that transgene expression and activity was restored in vivo. Studies are ongoing to complete the preclinical efficacy and safety of regulated LVV for X-CGD gene therapy before proceeding to clinical application.

ABSTRACT N. 35

HSR-TIGET - Other Genetic Diseases		
Principal Investigator	**BIFFI ALESSANDRA**	
Telethon grant N.	**TGTGSK07**	
Costs incurred €	**239.400**	
Centres: **1**	*Duration (yrs):* **-**	*Starting year:* **2011**

HEMATOPOIETIC STEM CELL GENE THERAPY FOR THE TREATMENT OF GLOBOID CELL LEUKODYSTROPHY

Ungari Silvia (1,2), Morena Francesco (3), Visigalli Ilaria (1), Gentner Bernhard (1), Delai Stefania (1), Martino Sabata (3), Naldini Luigi (1,2), Biffi Alessandra (1,4,5)

(1) San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, 20132, Milan, Italy, Tel. 0226434678; Fax 0226434668; E-mail: a.biffi@hsr.it
(2) Vita-Salute San Raffele University, San Raffaele Scientific Institute, Milano, Italy
(3) Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy
(4) HSR-TIGET Pediatric Clinical Research Unit, San Raffaele Scientific Institute, Milano, Italy
(5) Pediatric Immunohematology, San Raffaele Scientific Institute, Milan, Italy

Globoid Cell Leukodystrophy (GLD) or Krabbe disease is a rare and severe demyelinating lysosomal storage disorder due to the deficiency of galactocerebrosidase (GALC). Our research is aimed at improving the limited therapeutic benefit of hematopoietic stem cell (HSC) transplantation in GLD patients by means of lentiviral vector (LV) mediated gene transfer into autologous HSCs. Differently from what observed in other LSDs, in the case of GLD HSC gene therapy required a regulation of enzyme expression in HSCs due a toxic effect of GALC over-expression in the HSC compartment, and not in more differentiated cells, at least in the murine setting. To achieve such regulation we employed a GALC-encoding LV regulated by a micro RNA expressed only in HSCs (micro RNA 126). We proved the efficacy of this vector construct in vitro and also in vivo in the disease mouse model, of which we substantially improved survival and phenotype. Efficacy of HSC gene therapy was dose dependent and substantially greater than efficacy of normal donor HSC transplantation. To move forward these promising results, we are currently working at identifying the best LV design for a proper clinical translation. We cloned a codon optimized human GALC cDNA, allowing for an improved enzyme activity in respect to wild type levels, into a lentiviral backbone containing or not miRNA regulatory elements. These LVs were tested on human HSCs from healthy donors' cord blood and bone marrow. A sustained over-expression of GALC in the transduced HSC progeny after 2 weeks of in vitro culture, and a consistent miRNA126-mediated repression of activity one day post transduction were observed. However, no significant GALC-overexpression related toxicity was observed upon transduction with the GALC encoding vectors, even in the absence of regulation, neither in vitro nor in vivo, upon transplantation in immunodeficient mice. These findings suggest that human cells could be less sensitive to GALC over-expression related toxicity as compared to murine HSCs, even if direct testing of GALC-/- cells has still to be performed. In order to choose the safest LV construct we are currently aiming at the identification of markers involved in the apoptotic/inflammatory pathways induced by GALC over-expression by wide-transcriptome gene expression analysis on human and murine transduced cells. Moreover, ceramide and different sphingolipids, that are products of GALC-mediated enzymatic reactions and well-known anti- or pro-apoptotic stimuli, will be quantified on the same samples.

DULBECCO TELETHON INSTITUTE

ABSTRACT N. 36

DTI - Neuromuscular Diseases		
Principal Investigator	**CECCONI FRANCESCO**	
Telethon grant N.	**GGP10225**	
Total budget approved €	**532.500**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2010**

ROLE OF AUTOPHAGY IN MUSCLE DISEASES

Grumati Paolo (3), Di Bartolomeo Sabrina (1), Sferra Antonella (4), Nazio Francesca (2), Cianfanelli Valentina (1), Bertini Enrico (4), Bonaldo Paolo (3), Cecconi Francesco (1,2)

(1) Dulbecco Telethon Institute, Department of Biology, University of Rome Tor Vergata, and
(2) IRCCS Fondazione S. Lucia, Via del Fosso di Fiorano, 00142, Rome; Tel. +39.06.72594230; +39.06.501703092; Fax +390672594222, E-mail: francesco.cecconi@uniroma2.it
(3) Department of Biomedical Sciences, University of Padova, Padova, Italy
(4) Department of Neuroscience, Neuromuscular Unit, "Bambino Gesù" Childrens Hospital, Rome, Italy

Autophagy is a degradative process of bulk cytoplasm, long-lived proteins and entire organelles. Most Atg hortologs, such as Beclin 1, Atg5, Atg7, Vps34 and Atg12 as well as other Beclin 1-regulating factors, such as Ambra1, have been isolated in mammals and their inactivation in mouse revealed interesting embryonic phenotypes (Fimia GM et al., 2007). Ambra1 forms a stable complex with Beclin 1 and Vps34, and this complex is translocated from the cytoskeleton to the endoplasmic reticulum, during the early phases of autophagy induction, where it mediates autophagosome nucleation (Di Bartolomeo et al., 2010). A proper regulation of the autophagy flux is fundamental for the homeostasis of skeletal muscles during physiological situations and alterations of the flux are responsible of muscle disorders. Very recently, we have performed a histological analysis on Ambra1-deficient mouse muscles. We observed a clear muscle phenotype in 13.5 d.p.f. Ambra1gt/gt embryos. Developing muscles of Ambra1gt/gt embryos reveal a marked disorganization of muscle architecture. Myotubes and myofibrils do not follow a clear and orderly patter typical of skeletal muscles. The nascent muscles present an iper-cellularity that contributes to the skewed morphological structure and to the increased abnormal muscle dimension. To better elucidate Ambra1 function in muscle homeostasis we are generating the conditional knockout mouse, which will permit us to ablate the protein selectively in skeletal muscle.
The team also handles another mouse model for autophagy deregulation and genetic diseases: the collagen VI null mouse (a model for Bethlem myopathy and Ullrich congenital muscular dystrophy). We described that collagen VI null (Col6a1–/–) mice display a myopathic phenotype with accumulation of dysfunctional organelles and spontaneous apoptosis due to defective autophagy (Grumati et al., 2010). Col6a1–/– skeletal muscles display an impairment of autophagy flux. Forced activation of autophagy by dietary and pharmacological approaches ameliorates the dystrophic phenotype of Col6a1–/– mice. Furthermore, muscle biopsies from patients affected by Bethlem myopathy and Ullrich congenital muscular dystrophy showed reduced levels of Beclin 1 and Bnip3. We also demonstrated that a proper autophagy flux is fundamental for the homeostasis of skeletal muscles in response to physical exercise. Physical training exacerbated the dystrophic phenotype of Col6a1–/– mice, where autophagy flux is compromised. In a situation of altered autophagy, physical exercise seems to have more detrimental than beneficial effects (Grumati et al., 2011).
In addition, we are taking advantage from the Neuromuscular Unit of the Bambino Gesù Research Hospital in Rome, which has collected in the last years muscle biopsies and fibroblast cultures of patients affected by myopathies showing an increased muscle autophagy. Among these, two patients have been recently analyzed affected by Vici Syndrome, an inherited multisystemic disorder caused by a mutation in the KIAA1632 gene (epg5 human homolog), encoding for a key regulator of the autophagolysosome formation. We have observed that patients affected by Vici Syndrome show a marked fiber atrophy in the skeletal muscle and fiber hypotrophy and diameter variability with upregulation of p62, Nbr1 and LC3II, suggesting the involvement of epg5 in the degradation of myofibrillar components and demonstrating that Vici Syndrome is associated with defective autophagy (Cullup T et al., in press).

ABSTRACT N. 37

DTI - Neuromuscular Diseases		
Principal Investigator	**SANDRI MARCO**	
Telethon grant N.	**TCR09003**	
Total budget approved €	**610.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2010**

DEFINING THE MOLECULAR SIGNATURE OF MUSCLE WASTING. IDENTIFICATION OF THERAPEUTIC TARGETS TO COUNTERACT MUSCLE DEGENERATION

Sandri Marco (1,2), Nascimbeni Anna Chiara (3), Fanin Marina (3), Angelini Corrado (3)

(1) Dulbecco Telethon Institute at Venetian Institute of Molecular Medicine, via Orus 2, 35129 Padova, Tel. 0497923258, Fax 0497923250, E-mail: marco.sandri@unipd.it
(2) Department of Biomedical Science, University of Padova
(3) Department of Neurosciences, University of Padova, Biomedical Campus "Pietro d'Abano", Padova, Italy

Regulated removal of proteins and organelles by autophagy-lysosome system is critical for muscle homeostasis. Excessive activation of autophagy-dependent degradation contributes to muscle atrophy and cachexia. Conversely, inhibition of autophagy causes accumulation of protein aggregates and abnormal organelles, leading to myofiber degeneration and myopathy. Defects in lysosomal function result in severe muscle disorders such as Pompe (GSDII) disease, characterised by an accumulation of autophagosomes. However, whether autophagy is detrimental or not in muscle function of Pompe patients is unclear. We studied infantile and late-onset GSDII patients and correlated impairment of autophagy with muscle wasting. We also monitored autophagy in patients who received recombinant a-glucosidase. Our data show that infantile and late-onset patients have different levels of autophagic flux, accumulation of p62-positive protein aggregates and expression of atrophy-related genes. Although the infantile patients show impaired autophagic function, the late-onset patients display an interesting correlation among autophagy impairment, atrophy and disease progression. Moreover, reactivation of autophagy in vitro contributes to GAA maturation in both healthy and diseased myotubes. Together, our data suggest that autophagy protects myofibers from disease progression and atrophy in late-onset patients.

ABSTRACT N. 38

DTI - Neuromuscular Diseases		
Principal Investigator	**ORLANDO VALERIO**	
Telethon grant N.	**TCR11001**	
Total budget approved €	**200.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2011**

ROLE OF THE EPIGENOME AND NONCODING GENOME IN DUCHENNE MUSCLE DYSTROPHY

Bodega Beatrice (1), Della Valle Francesco (1), Saccone Valentina (2), Consalvi Silvia (2), Martone Julie (3), Cazzella Valentina (3), Hayashizaki Yoshihide (4), Carninci Piero (4), Forrest Al (4), Bozzoni Irene (3), Puri Pier Lorenzo (2), Orlando Valerio (1)

(1) Dulbecco Telethon Institute (DTI), IRCSS Fondazione Santa Lucia, Epigenetics and Genome reprogramming, Via del Fosso di Fiorano 64, 00143 Rome, Italy, Tel 06 501703262, E-mail: vorlando@dti.telethon.it
(2) Dulbecco Telethon Institute (DTI), IRCCS Fondazione Santa Lucia, Phamacology and Epigenetics, Rome, Italy
(3) Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Italy
(4) RIKEN Yokohama Institute, Omics Science Center, Yokohama, Kanagawa, Japan

Epigenetic regulation and impact of repetitive elements (45% of genome) in human genome function and disease is largely unknown. Recent reports indicate that LINE-1 (L1) dynamic activity occurs in brain cells and defects in their mobilization and epigenome regulation are associated with neurological disorder. Whether somatic L1 retrotransposition regulation could contribute to other differentiation programs and impact non-neurological, diseases is currently unexplored.
Duchenne muscular dystrophy (DMD) is a genetically well defined disease being associated with mutations in the dystrophin gene. Increasing evidence indicates that disruption of the dystrophin-associated protein complex (DAPC) at the sarcolemma affects not only the structure of muscle fibers4, but impact global genome expression (coding and non coding transcripts) through deregulation of the nNOS-HDAC2 pathway. Here we show that during skeletal myogenesis LINE-1 (L1) transcription and copy number variation (CNV) are dynamically regulated while in DMD cells HDAC2 is aberrantly recruited at L1 promoter and their mobilization is impaired, suggesting a role for L1 activity in muscle cell differentiation. Indeed, we found that inhibition of L1 mobilization by reverse transcriptase impaired the differentiation ability of normal human primary muscle cells. Notably, functional restoration of dystrophin-nNOS-HDAC2 signaling and fiber functionality by HDAC inhibitors or dystrophin re-expression by exon-skipping could restore normal L1 expression levels and CNV either in the mdx mice and in DMD primary muscle cells. These results uncover the epigenetic regulation of L1 sequences as a direct target of dystrophin signaling to HDACs in muscle cell progenitors, deregulated in DMD patients and highly responsive to therapeutic strategy currently in clinical trials. We propose that repetitive elements could be potential players in cell differentiation, and their deregulation a key epigenetic trait in degenerative diseases.

ABSTRACT N. 39

DTI - Neuromuscular Diseases		
Principal Investigator	**GABELLINI DAVIDE**	
Telethon grant N.	**TCR11003**	
Total budget approved €	**210.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

FSHD MUSCULAR DYSTROPHY REGION GENE 1 (FRG1) BINDS SUV4-20H1 HISTONE METHYLTRANSFERASE AND IMPAIRS MYOGENESIS

Neguembor Maria Victoria (1,2), Xynos Alexandros (1), Onorati Maria Cristina (3), Caccia Roberta (1), Bortolanza Sergia (1), Godio Cristina (1), Corona Davide F. (3), Schotta Gunnar (4), Gabellini Davide (1)

(1) Dulbecco Telethon Institute and Division of Regenerative Medicine, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano Tel. 02-26435934 Fax 02-26435544, E-mail: gabellini.davide@hsr.it
(2) Università Vita-Salute San Raffaele, Milan, Italy
(3) Dulbecco Telethon Institute, Sezione Biologia Cellulare, Dipartimento STEMBIO, Università degli Studi di Palermo, Italy
(4) Munich Center for Integrated Protein Science and Adolf Butenandt Institute, Ludwig Maximilians University, Munich, Germany

Facioscapulohumeral Muscular Dystrophy (FSHD) is an autosomal dominant myopathy with a strong epigenetic component. It is associated with deletion of the D4Z4 macrosatellite repeat leading to over-expression of nearby genes. Among them, we focused on FSHD Region Gene 1 (FRG1) since its over-expression in mice, X. laevis and C. elegans leads to FSHD-like defects. Here we show that FRG1 binds and interferes with the activity of the histone methyltransferase Suv4-20h1 both in mammals and in Drosophila.
Myogenic defects have been widely reported in FSHD but the molecular mechanism is unknown. Interestingly, FRG1 over-expression or Suv4-20h1 knockdown inhibit myogenesis. Moreover, Suv4-20h KO mice develop muscular dystrophy. Finally, we recognize the FRG1/Suv4-20h1 target Eid3 as a novel myogenic inhibitor that contributes to the identified muscle differentiation defects and is inappropriately up-regulated selectively in FSHD patients. Our study suggests a novel role of FRG1 as epigenetic regulator of muscle differentiation, indicates that Suv4-20h1 has a gene-specific function in myogenesis and discover potential therapeutic targets for FSHD.

ABSTRACT N. 40

DTI - Neuromuscular Diseases		
Principal Investigator	**ZITO ESTER**	
Telethon grant N.	**TCP12001**	
Total budget approved €	**517.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2013**

DISSECTING THE MOLECULAR BASIS OF SEPN1-RELATED-MYOPATHIES

Zito Ester

Dulbecco Telethon Institute, Istituto di Ricerche Farmacologiche Mario Negri, Via G. la Masa 19, 20156 Milano, Italy, Tel. +39.02.39014402, Fax +39.02.3546277, E-mail: ester.zito@marionegri.it

Selenoprotein N (SEPN1), a member of the selenocysteine-containing protein family, is localized in the endoplasmic reticulum (ER) and ubiquitously expressed throughout the body. However, the complete loss of SEPN1 gene function leads to a selective myopathy phenotype (OMIM 606210, rigid spine muscular dystrophy and SEPN1-related myopathies), which suggests that, despite its widespread expression, SEPN1 is particularly involved in molecular processes relevant to muscle physiology.
Although the precise function of SEPN1 remains unknown, the presence of a thioredoxin reductase domain in the core of the protein indicates a redox function. Because of recent data have shown that

this protein plays a central role in regulating cellular redox homeostasis, the redox manipulation of SEPN1 may be advantageous for functional studies aimed at revealing novel therapeutic targets.
As redox changes in the ER have been associated with altered calcium handling and muscle dysfunction, the working hypothesis of this proposal is that the redox activity of SEPN1 may regulate the function of key proteins involved in calcium flux and signalling.
Finally, emerging evidence suggests some compounds as reductants of new client proteins in the ER. It may thus be well placed to compensate for the lack of SEPN1 in the ER and merits evaluation as a potential treatment of SEPN1-related myopathies in an ad hoc mice model.

ABSTRACT N. 41

DTI - Neuromuscular Diseases		
Principal Investigator	**BONETTO VALENTINA**	
Telethon grant N.	**TCR08002**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2009**

IMPAIRMENT OF CYCLOPHILIN A LINKS SOD1 AND TDP-43 PATHOLOGIES THROUGH HNRNP COMPLEX DESTABILIZATION IN AMYOTROPHIC LATERAL SCLEROSIS

Lauranzano Eliana (1), Stucchi Riccardo (1), Pasetto Laura (1), Pozzi Silvia (1), Massignan Tania (1), Bendotti Caterina (2), Bonetto Valentina (1)

(1) Dulbecco Telethon Institute, Istituto di Ricerche Farmacologiche Mario Negri, Dipartimento di Biochimica e Farmacologia Molecolare, Laboratorio di Proteomica Traslazionale, Via La Masa 19, 20156 Milano, Italy, Tel. +39.02.39014548, Fax +39.02.39014744, E-mail: valentina.bonetto@marionegri.it
(2) Istituto di Ricerche Farmacologiche Mario Negri, Dipartimento di Neuroscienze

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease affecting preferentially motor neurons. Cyclophilin A (CypA) was identified as a hallmark of disease in mutant SOD1 (mSOD1) animal models of familial ALS (fALS) at a presymptomatic stage, and in sporadic (sALS) patients (Massignan et al. Biochem Biophys Res Commun 2007, 353, 719-725; Nardo et al. PLoS One, 2011, 6, e25545). Moreover, CypA was enriched in the spinal cord aggregates of mSOD1 mice and sALS patients (Basso et al. PLoS One, 2009, 4, e81303). CypA is an ubiquitous protein with multiple functions relevant to the central nervous system, where it is not known its specific function and is abundantly expressed. Insights into CypA function in ALS were provided via a proteomic analysis of its interacting proteins, that functionally associated CypA with different proteins networks. In particular, it extensively binds proteins regulating RNA metabolism, including several hnRNPs and TDP-43, a major disease protein in ALS. TDP-43 and CypA interact in the nucleus, in an RNA-dependent way. CypA has a key role in the stabilization of TDP-43/hnRNP A2/B1 interaction, and TDP-43-mediated HDAC6 expression regulation, properties impaired in TDP-43 ALS-mutants, possibly because of a loss-of-interaction with CypA. CypA interacts also with mSOD1, suggesting a gain-of-interaction specifically linked to fALS. Mice expressing mSOD1 and lacking CypA show increased levels of insoluble mSOD1 and hyperphosphorylated TDP-43 in the spinal cord at the onset. This work shows that CypA has a protective role in ALS: as a chaperone (for mSOD1) and in maintenance of multi-protein (TDP-43/hnRNPs) complex stability. Regardless the cause of the disease, mSOD1 or alterations in TDP-43, the interaction with CypA is impaired and it is co-sequestered in proteinaceous aggregates, altering its protective activities. The net effect is the formation of pathological inclusions that may lead to a compromised RNA metabolism. CypA being a key interacting partner of both mSOD1 and TDP-43 can represent the "missing link" of these two patho-mechanisms in ALS and an interesting target for therapeutic interventions.

ABSTRACT N. 42

DTI - Neuromuscular Diseases		
Principal Investigator	**PENNUTO MARIA**	
Telethon grant N.	**TCP12013**	
Total budget approved €	**517.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2013**

TARGETING AKT SIGNALING IN MUSCLE TO IDENTIFY NEW THERAPEUTIC STRATEGIES FOR SPINAL AND BULBAR MUSCULAR ATROPHY

Rocchi Anna (1), Pellegrini Matteo (1), Urciolo Anna (2), Blaaw Bert (3), Bonaldo Paolo (2), Sandri Marco (4), Pennuto Maria (1)

(1) Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, via Morego 30, Genoa 16163, Italy, Tel. +39.010.71781793, Fax +39.010.71781230
(2) Departments of Histology Microbiology & Medical Biotechnologies, University of Padua, Italy
(3) Dipartimento di Anatomia e Fisiologia Umana, University of Padua, Italy
(4) VIMM, University of Padua, Italy

The insulin-like growth factor 1 (IGF-1)/Akt pathway plays a critical role in the maintenance of muscle homeostasis in adulthood. IGF-1 induces muscle hypertrophy by activating pathways that lead to new protein synthesis, and this occurs through a cascade of phosphorylation events that lead to inhibition of Gsk3beta and activation of mTOR. On the other hand, IGF-1/Akt inhibits muscle atrophy through inactivation of FOXO3a, which in turn stimulates protein degradation via both the ubiquitin-proteasome system and autophagy.
Spinal and bulbar muscular atrophy (SBMA) is a motor neuron disease characterized by the degeneration of motor neurons from brainstem and spinal cord. SBMA is caused by expansion of a polyglutamine (polyQ) tract in the gene coding for androgen receptor (AR). Patients develop progressive face and limb skeletal muscle fasciculation, weakness, and atrophy. Emerging evidence supports the concept that muscle atrophy in SBMA results not only from denervation, but also from direct myopathic changes exerted by mutant AR in skeletal muscle. Consistent with a critical role of muscle in SBMA pathogenesis, we have previously shown that overexpression of IGF-1 selectively in the skeletal muscle of SBMA mice leads to activation of Akt, phosphorylation and degradation of mutant AR by the proteasome, and attenuation of disease manifestations (Palazzolo et al., 2009). These results indicate that skeletal muscle is an important component of disease pathogenesis and a valuable therapeutic target to develop intervention.
Here, we propose to test the hypothesis that the IGF-1/Akt pathway is altered in the muscle of SBMA mice. To address this question, we used the knock in mouse model of SBMA generated by Dr Lieberman (Michigan University, USA). We found that Akt expression levels and phosphorylation are increased in skeletal muscle, but not spinal cord, of SBMA mice. Importantly, we found that the Akt-dependent pathways leading to new protein synthesis and macro-autophagy are activated in SBMA muscle. Notably, activation of the pathways leading to new protein synthesis occurs earlier that those leading to macro-autophagy, which we show is defective at late stages of disease. We propose that mutant AR-induced atrophy results in compensatory hypertrophy in the adult SBMA mouse, which occurs through induction of Akt-dependent activation of new protein synthesis. Unbalance between new protein synthesis and defective macro-autophagy may lead to skeletal muscle atrophy in SBMA.

ABSTRACT N. 43

DTI - Neuromuscular Diseases		
Principal Investigator	**BOLINO ALESSANDRA**	
Telethon grant N.	**GGP12017**	
Total budget approved €	**253.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

PHOSPHOLIPID METABOLISM AND MEMBRANE TRAFFICKING IN THE PATHOGENESIS OF CHARCOT-MARIE-TOOTH NEUROPATHIES

Françoise Piguet (1), Ilaria Vaccari (1), Stefania Scarlino (1), Angela Cattaneo (2), Bernard Payrastre (3), Michael Simons (4), Angela Bachi (2), Alessandra Bolino (1)

(1) Dulbecco Telethon Institute, ISNPE-Division of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58 20132, Milan, Italy, Tel. 02.26434743, Fax 02.26435093, E-mail: bolino.alessandra@hsr.it
(2) Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy
(3) INSERM U1048, Toulouse, France
(4) Max Planck Institute for Experimental Medicine, Goettingen, Germany

Charcot-Marie-Tooth 4B1 (CMT4B1) is a severe autosomal recessive demyelinating neuropathy, characterized by formation of redundant

myelin sheaths (myelin outfolding) that likely degenerate causing demyelination and axonal problems. We previously reported that CMT4B1 is caused by loss of MTMR2 in human (Bolino et al., 2000) and created a faithful mouse model of the disease (Bolino et al., 2004; Bolis et al., 2005). MTMR2 is a phospholipid phosphatase with a suggested role in intracellular trafficking (Hnia, Vaccari, Bolino and Laporte, Trends in Molecular Medicine 2012). We recently identified a potential mechanism using in vivo and in vitro models (Schwann cell/DRG neuron cocultures from Mtmr2-null mice (Bolis et al., 2009) and proposed that Mtmr2 belongs to a molecular machinery that titrates membrane formation during myelination. According to this model, myelin outfoldings arise as a consequence of the loss of negative control on the amount of membrane produced during myelination. However, the physiological event regulated by MTMR2 and whether this function is mediated by the PI phosphatase activity in vivo still remain to be defined.
In this project we will investigate if MTMR2 plays a role in clathrin-dependent and independent endocytosis and if defects in this pathway are at the basis of the CMT4B1 pathogenesis. Following the hypothesis that MTMR2 is required for receptor downregulation, we will analyze the impact of MTMR2 loss on EGFR and erbB2 receptors trafficking, as the NRG1-erbB pathway promotes myelination in the PNS (AIM1).
Moreover, we recently found that catalytically inactive MTMR2-C417S rescues myelin outfoldings in Mtmr2-null co-cultures as the wild type protein. This result apparently contradicts the hypothesis that MTMR2 is a PI phosphatase. We will therefore determine the rescuing potential in Mtmr2-null co-cultures of MTMR2 proteins carrying missense mutations that should interfere with substrate recognition or alter its catalytic activity. We will also correlate the rescue or not with PI dosages (AIM2).
Finally, we will explore a therapeutical approach aimed at rescuing myelin outfoldings by rebalancing PI levels. We will specifically target PtdIns(3,5)P2 by inhibition of PIKfyve, the enzyme that produces PtdIns(3,5)P2 from PtdIns3P. We recently provided evidence that imbalance of PtdIns(3,5)P2 in Schwann cells causes myelin oufoldings in Mtmr2-null nerves (Vaccari et al., PLoS Genetics 2011). We will exploit the use of the PIKfyve specific inhibitor YM201636 in vivo by intrathecal injection to ameliorate the neuropathy of Mtmr2-null mice (AIM3).

ABSTRACT N. 44

DTI - Neuromuscular Diseases		
Principal Investigator	**CHIESA ROBERTO**	
Telethon grant N.	**TCR08005**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2009**

CELLULAR MECHANISMS OF SYNAPTIC DYSFUNCTION IN INHERITED PRION DISEASES

Senatore Assunta (1), Colleoni Simona (2), Verderio Claudia (3), Restelli Elena (1), Morini Raffaella (3), Condliffe Steven B. (3), Bertani Ilaria (1), Mantovani Susanna (1), Canovi Mara (2), Micotti Edoardo (2), Forloni Gianluigi (2), Dolphin Annette C. (4), Matteoli Michela (3), Gobbi Marco (2), Chiesa Roberto (1)

(1) Dulbecco Telethon Institute c/o Mario Negri Institute for Pharmacological Research, via G. La Masa 19 20156 Milan, Italy, Tel. +39.0239014428, E-mail: roberto.chiesa@marionegri.it
(2) Mario Negri Institute for Pharmacological Research, Milan, Italy
(3) University of Milan, Italy
(4) University College London, Uk

How mutant prion protein (PrP) leads to neurological dysfunction in genetic prion diseases is unknown. Tg(PG14) mice synthesize a misfolded mutant PrP which is partially retained in the neuronal endoplasmic reticulum (ER). As these mice age, they develop ataxia and massive degeneration of cerebellar granule neurons. Here we report that motor behavioral deficits in Tg(PG14) mice emerge before neurodegeneration and are associated with defective glutamate exocytosis from granule neurons due to impaired calcium dynamics. We found that PrP interacts with the voltage-gated calcium channel alpha2delta-1 subunit which promotes the anterograde trafficking of the channel. Owing to ER retention of mutant PrP, alpha2delta-1 accumulates intracellularly, impairing delivery of the channel complex to the cell surface. Thus mutant PrP disrupts cerebellar glutamatergic neurotransmission by reducing the number of functional channels in cerebellar granule neurons. These results link intracellular PrP retention to synaptic dysfunction, indicating new modalities of neurotoxicity and potential therapeutic strategies.

[Senatore et al., Mutant PrP suppresses glutamatergic neurotransmission in cerebellar granule neurons by impairing the membrane delivery of VGCC alpha2delta-1 subunit. Neuron, 74:300-313, 2012].

ABSTRACT N. 45

DTI - Neuromuscular Diseases		
Principal Investigator	**PASSAFARO MARIA PIA**	
Telethon grant N.	**GGP12097**	
Total budget approved €	**255.400**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2012**

ANALYSIS OF NEURONAL ALTERATIONS ASSOCIATED TO TM4SF2 MUTATIONS AND THEIR RESCUE BYGENETIC AND PHARMACOLOGICAL THERAPIES

Folci Alessandra (1), Murru Luca (1), Vezzoli Elena (2), D'Adamo Patrizia (3,4), Francolini Maura (2,5), Passafaro Maria (1,3)

(1) CNR Neuroscience Institute, Via Vanvitelli 32, 20129, Milan, Italy, Tel.+039.02.50317102, Fax +039.02.7090574, E-mail: m.passafaro@in.cnr.it
(2) Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milan, Italy
(3) DTI Dulbecco Telethon Institute
(4) Vita-Salute San Raffaele University, Milan, Italy
(5) Fondazione Filarete, Milan, Italy

TM4SF2 is a gene involved in intellectual disability (ID) that encodes the tetraspanin TSPAN7. Tetraspanins are evolutionarily-conserved membrane proteins that associate dynamically with numerous partner proteins in tetraspanin enriched microdomains (TEMs) and regulate cell morphology, motility, and signaling. TM4SF2 is mainly expressed in brain. We recently found that TSPAN7 regulates synapse development and AMPA receptor trafficking in cultured hippocampal neurons. In fact, TSPAN7 silencing reduces the size and stability of dendritic spines, preventing their remodelling upon cLTP induction and impairs the expression of synaptic markers. We also identified PICK1 (protein interacting with C kinase 1), a protein involved in the internalization and recycling of AMPA receptors (AMPAR), as a novel partner of TSPAN7. TSPAN7 silencing by shRNA, promotes GluA2/3 internalization in a PICK1-dependent manner, and specifically reduces postsynaptic AMPAR transmission.
Starting from these data, we are analyzing the function of this protein in TM4SF2 KO mice. We first analysed the anatomy of the CNS in young and adult KO mice, focusing on the organization of hippocampus and cortex and found that no differences can be found between wild type and mutant animals at least at the level of optical microscopy, thus we are now analysing the ultrastructure of neurons in the same areas to look for differences in the fine structure of synapses. Moreover, in preliminary results we found a significant alteration in frequency of mEPSCs in hippocampus of KO mice compared to wild type animals. If the animal will reproduce the cognitive impairment found in humans with ID, it could represent a useful model for pharmacological screening.

ABSTRACT N. 46

DTI - Neuromuscular Diseases		
Principal Investigator	**D'ADAMO PATRIZIA**	
Telethon grant N.	**TCR11002**	
Total budget approved €	**470.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

ANALYSIS OF RAB39B ROLE IN X-LINKED INTELLECTUAL DISABILITY: AMPA RECEPTORS AS POSSIBLE THERAPEUTIC TARGETS

Giannandrea Maila (1), Mignogna Maria Lidia (1,2), Magro Daniela (2), Bassani Silvia (3), Mapelli Lisa (3), Passafaro Maria (3), Esteban Jose Antonio (4), D'Adamo Patrizia (1,2)

(1) DTI at San Raffaele Scientific Institute, via Olgettina 58 Milan, Italy Tel. 02.26434604, Fax 02.23464844, E-mail: p.dadamo@hsr.it
(2) Università Vita-Salute San Raffaele, Milan, Italy
(3) CNR, Neuroscience Institute, Milan, Italy
(4) Universidad Autonoma de Madrid, Madrid, Spain

Intellectual Disability (ID) is a common developmental brain disorder characterized by an IQ lower than 70. ID affects about 2% of

the population and the disease can be comorbid with autistic spectrum disorders with a very severe social and economic impact.
RAB GTPases function are a class of proteins playing a key role in neuronal release machinery and synaptic maturation and control intracellular trafficking by acting as molecular switches between an active (GTP-bound) and an inactive (GDP-bound) conformation.
We have identified mutations in RAB39B gene in XLID families. Our studies show that RAB39B is a neuronal specific gene and that the in vitro down regulation of its expression causes a drastic reduction in the number of synapses, a phenotype that could correlate with the ID characteristics. Searching for RAB39B interacting proteins, we have established that RAB39B binds GM130 and Trip11, two Golgi structural proteins, Dynactin1, a protein that modulates Dynein binding to organelles and microtubules and is involved in cargo transport into dendrites.
The main RAB39B-GTP interacting protein is PICK1 (protein interacting with C-kinase1), that plays important roles in synaptic AMPA receptors subunits (GluA2/3) surface expression, trafficking and synaptic targeting in post-synaptic site. Electrophysiology work demonstrates that downregulation in RAB39B expression is affecting both pre and post-synaptic function. We focused on post-synaptic function where RAB39B plays a dynamic role in the cargo transport of GluA2/3 AMPA subunits: they bind the PDZ domain of PICK1 to exit from the endoplasmic reticulum and reach the Golgi apparatus and the microtubule minus-end directed motor protein Dynein which is essential for the polarized sorting of GluA2. To demonstrate this we first determined possible PICK1 and GluA2 alterations in their cellular localization by immunofluorescence experiments on Rab39b-silenced primary hippocampal neurons, finding that PICK1 is mislocalized and that the amount of GluA2 exposed on the neuronal membrane is decreased, while the amount of GluA1 subunit increases.
Studies on organotypic mouse hippocampal slices have confirmed this observation and revealed that down regulated RAB39B causes no changes in basal AMPA currents and LTD, but increases the rectification index because it increases the fraction of GluA2 subunit-lacking AMPARs at synapses, and supporting the hypothesis that RAB39B is necessary to deliver (or maintain) GluA2-containing receptors at the postsynaptic membrane.
Since the combination of in vitro results with the extensive cellular, biochemical and behavioral analysis carried out on an animal model is fundamental to understand the RAB39B function in synaptic plasticity and cognition, we generated a Rab39b down regulated mouse. Current characterization of this animal model is establishing if the observed in vitro synaptic changes can be reproduced and behavioural studies are characterizing the associated cognitive impairments. This animal model may provide a clear advantage to discover new therapeutic strategies targeting a deficit in AMPA receptor function.

ABSTRACT N. 47

DTI - Neuromuscular Diseases		
Principal Investigator	**SCORRANO LUCA**	
Telethon grant N.	**GGP12162**	
Total budget approved €	**371.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

EXTENDING THE OPTIC ATROPHY 1 DEPENDENT CRISTAE REMODELING: FROM MODELS TO A RATIONALE FOR THERAPY OF AUTOSOMAL DOMINANT OPTIC ATROPHY

Scorrano Luca

Dulbecco-Telethon Institute, Venetian Institute of Molecular Medicine, Via Orus 2, 35129 Padova, Italy, Tel. +39.049.7923221, Fax +39.049.7923256, E-mail: lscorrano@dti.telethon.it

Autosomal dominant optic atrophy (ADOA) is caused by mutations in Optic Atrophy 1 (OPA1), a dynamin-related protein of the inner mitochondrial membrane. During my tenure as a Scientist of the DTI, we clarified that OPA1 is a multifunctional protein participating in genetically distinct pathways of mitochondrial fusion and cristae remodelling, both impaired by pathogenetic mutations. We extended our investigation on the (dys)function of OPA1 and our preliminary results indicate that (i) OPA1 is a key modulator of apoptosis and autophagy in vivo; (ii) OPA1 is a master regulator of mitochondrial cristae architecture, impacting on respiratory chain supercomplex assembly and mitochondrial metabolism; (iii) increased autophagy in axons of retinal ganglion cells carrying pathogenic OPA1 depletes them of mitochondria; (iv) OPA1 resides in multimolecular complexes that comprise potential key regulators of its multiple functions. We therefore hypothesize that by engaging in interactions with different partners, OPA1 regulates mitochondrial functions. Its mutations increase autophagy and susceptibility to apoptosis, especially in RGCs. These multiple regulatory points offer several potential targets for therapeutic strategies that can interfere with the natural course of the disease.
In order to verify our hypothesis, we plan to combine genetics, biochemistry, imaging, mitochondrial physiology, proteomics to address: (i) how OPA1 regulates mitochondrial metabolism from the cristae; (ii) how changes in OPA1 levels and function impinge on autophagy, especially in RGC; (iii) if the changes in mitochondrial metabolism and autophagy can be exploited therapeutically in vitro and in vivo. This integrated approach aims at unraveling the pathogenesis of ADOA, and therefore to pave the way for its treatment. At the same time, we expect to clarify how mitochondria participate in key cellular metabolic and quality control processes.

ABSTRACT N. 48

DTI - Other Genetic Diseases		
Principal Investigator	**FANELLI FRANCESCA**	
Telethon grant N.	**GGP11210**	
Total budget approved €	**361.900**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2011**

INTEGRATED IN SILICO, IN VITRO, AND IN VIVO STUDIES TOWARDS THE DESIGN OF MOLECULES WITH THERAPEUTIC POTENTIAL FOR RETINITIS PIGMENTOSA

Felline Angelo (1,2), Behnen Petra (3), Marigo Valeria (3), Fanelli Francesca (1,2)

(1) Department of Life Sciences, University of Modena and Reggio Emilia, via Via Campi 183, 41125, Modena, Italy, Tel. +39.059.2055114, Fax +39.059.373543; E-mail: fanelli@unimo.it
(2) Dulbecco Telethon Institute (DTI)
(3) Department of Life Sciences, Via Campi 287, Modena

Retinitis Pigmentosa (RP) is a group of hereditary human diseases that are characterized by progressive retinal degeneration and severe visual impairment in as many as 1.5 million patients worldwide. Despite the high genetic heterogeneity of RP syndrome, ~140 point mutations were discovered in the rhodopsin gene, encoding the visual pigment molecule of rod cells that captures light and activates an electrical signal transmitted to the brain for vision. Rhodopsin belongs to the G protein Coupled Receptor superfamily of seven-transmembrane proteins [Fanelli F, De Benedetti PG (2011) Chem Rev 111: PR438-535]. Photon absorption by rhodopsin causes the cis-trans isomerization of the covalently bound retinal chromophore and the consequent formation of the signaling active state. The vast majority of the rhodopsin mutations cause the Autosomal Dominant form (ADRP) of the pathology. A recent analysis of published data indicates that 89% of the biochemically characterized rhodopsin mutants are misfolded, supporting the protein-misfolding disease model suitable for treatments with pharmacological chaperones, i.e. ligands able to rescue proper protein fold [Krebs MP et al. (2010) J Mol Biol 395: 1063-1078]. Yet, the structural features of such mutants are obscure, which hampers rational drug design.
This transdisciplinary project aims at integrating molecular simulations of ADRP rhodopsin mutants, virtual screening of compound libraries as well as in vitro and in vivo experiments to discover small therapeutic molecules able to recover rhodopsin folding.
We started from in silico and in vitro screening of 46 ADRP rhodopsin mutants known to hold variable defects in folding. In silico experiments on wild type and mutated rhodopsin consisted in thermal unfolding simulations combined with the graph-based Protein Structure Network analysis, in line with previous mechanical unfolding simulations [Fanelli F, Seeber M (2010) FASEB J 24: 3196-3209]. ADRP rhodopsin mutations share more or less marked abilities to impair selected highly connected nodes in the protein structure network, i.e. hubs, essentially located in the retinal binding site, which participates in the stability core of the protein. We could define a number of computational indices whose combination led to a structural classification of the mutants.
In parallel, the same mutants were cloned in expression vectors and expressed in vitro in COS-7 cells. The subcellular localization was analyzed with two monoclonal antibodies recognizing either the extracellular N-terminal or the intracellular C-terminal of rhodopsin. In order to define levels of expression and differences in post-translational modifications of the mutants compared to the wild type, the proteins were analyzed by Western blotting. These two levels of analysis allowed an in vitro characterization of the different mutants

that we are now integrating with the structural analysis to generate the first classification of ADRP rhodopsin mutants based on a multi-scale approach, i.e. at the cellular and atomic levels of detail. This knowledge will be our starting point for the choice of a number of mutations to be used to reveal the chaperone potential of small compounds selected by virtual and in vitro screening.

ABSTRACT N. 49

DTI - Other Genetic Diseases		
Principal Investigator	**MUSCO GIOVANNA**	
Telethon grant N.	**TCR07003**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2008**

NEW PERSPECTIVES IN THE STRUCTURAL APPROACH OF GENETIC DISEASES

Gaetani M (1), Matafora V (2), Spiliotopoulos D (1), Mollica L (1), Quilici G (1), Chignola F (1), Mannella V (1), Zucchelli C (1), Peterson P (3), Bachi A (2), Musco G (1)

(1) Dulbecco Telethon Institute c/o S. Raffaele Scientific Institute, Biomolecular NMR Laboratory, Via Olgettina 58 20132, Milan, Italy, Tel. +39.02.26434824, Fax +39.02.26434824, E-mail: gmusco@dti.telethon.it
(2) Biomolecular Mass Spectrometry Unit, San Raffaele Scientific Institute, Milan, Italy
(3) Department of Molecular Pathology, University of Tartu, Estonia

Mutations in autoimmune regulator (AIRE) gene cause autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. AIRE is expressed in thymic medullary epithelial cells, where it promotes the expression of peripheral-tissue antigens to mediate deletional tolerance, thereby preventing self-reactivity. AIRE contains two plant homeodomains (PHDs) which are sites of pathological mutations. AIRE-PHD fingers are important for AIRE transcriptional activity and presumably play a crucial role in the formation of multimeric protein complexes at chromatin level which ultimately control immunological tolerance. As a step forward the understanding of AIRE-PHD fingers in normal and pathological conditions, we investigated their structure and used a proteomic SILAC approach to assess the impact of patient mutations targeting AIRE-PHD fingers. Importantly, both AIRE-PHD fingers are structurally independent and mutually non-interacting domains. In contrast to D297A and V301M on AIRE-PHD1, the C446G mutation on AIRE-PHD2 destroys the structural fold, thus causing aberrant AIRE localization and reduction of AIRE target genes activation. Moreover, mutations targeting AIRE-PHD1 affect the formation of a multimeric protein complex at chromatin level. Overall our results reveal the importance of AIRE-PHD domains in the interaction with chromatin-associated nuclear partners and gene regulation confirming the role of PHD fingers as versatile protein interaction hubs for multiple binding events.

ABSTRACT N. 50

DTI - Other Genetic Diseases		
Principal Investigator	**SETTEMBRE CARMINE**	
Telethon grant N.	**TCP12008**	
Total budget approved €	**517.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2013**

LYSOSOMAL CONTROL OF WHOLE BODY METABOLISM

Settembre Carmine (1,2,3,10), Rossella De Cegli (1), Gelsomina Mansueto (1), Pradip Saha (4), Francesco Vetrini (2,3), Orane Visvikis (5), Tuong Huynh (2,3), Annamaria Carissimo (1), Donna Palmer (2), Tiemo Jürgen Klisch (2,3), Amanda Wollenberg (5), Diego Di Bernardo (1,6), Lawrence Chan Chan (4,7,8), Javier Irazoqui (5), Andrea Ballabio (1,2,3,9)

(1) Telethon Institute of Genetics and Medicine (TIGEM), Via Pietro Castellino 111, 80131, Naples, Italy
(2) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA
(3) Jan and Dan Duncan Neurological Research Institute, Texas Children Hospital, Houston, Texas, USA
(4) Diabetes and Endocrinology Research Center, Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Baylor College of Medicine, Houston, Texas, USA
(5) Program in Developmental Immunology, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA
(6) Department of Systems and Computer Science, "Federico II" University of Naples, Naples, Italy
(7) Departments of Molecular & Cellular Biology and Biochemistry, Baylor College of Medicine
(8) St. Luke's Episcopal Hospital, Houston, Texas, USA
(9) Medical Genetics, Department of Pediatrics, Federico II University, Via Pansini 5, 80131 Naples, Italy
(10) Dulbecco Telethon Institute (DTI)

The lysosomal-autophagic pathway is activated by starvation and plays an important role in both cellular clearance and lipid catabolism. However, the transcriptional regulation of this pathway in response to metabolic cues is currently uncharacterized. Here we show that the transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy, is induced by starvation through an autoregulatory feedback loop and exerts a global transcriptional control on lipid catabolism via PGC1a and PPARa. Thus, during starvation a transcriptional mechanism links the autophagic pathway to cellular energy metabolism. The conservation of this mechanism in Caenorhabditis elegans suggests a fundamental role for TFEB in the evolution of the adaptive response to food deprivation.
Viral delivery of TFEB to the liver prevented weight gain and metabolic syndrome in both diet-induced and genetic mouse models of obesity, suggesting a novel therapeutic strategy for disorders of lipid metabolism.

ABSTRACT N. 51

DTI - Other Genetic Diseases		
Principal Investigator	**SERAFINI MARTA**	
Telethon grant N.	**TCP07004**	
Total budget approved €	**517.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2008**

EVALUATION OF STEM CELLS-MEDIATED GENE THERAPY FOR HURLER'S SYNDROME

Pievani Alice (1,2), Gatto Francesca (1,2), Sacchetti Benedetto (4), Redaelli Daniela (1,2), Biondi Andrea (2,3), Riminucci Mara (4), Bianco Paolo (4), Serafini Marta (1,2)

(1) Dulbecco Telethon Institute at Tettamanti Research Center, Pediatric Department, University of Milano-Bicocca, San Gerardo Hospital, via Pergolesi, 33 20900 Monza, Italy, Tel. +39.039.2332232, Fax: +39.039.2332167, E-E-mail: serafini.marta@gmail.com
(2) Centro Ricerca Tettamanti, Department of Pediatrics, University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy
(3) Department of Pediatrics, University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy
(4) Department of Molecular Medicine, La Sapienza University, Rome, Italy

Mucopolysaccharidosis type I (MPSIH; Hurler syndrome) is a rare genetic disorder caused by mutations in the IDUA gene, resulting in the deficiency of alpha-L-iduronidase (IDUA) enzyme activity with a consequent intracellular accumulation of glycosaminoglycans (GAG). Among a broad spectrum of clinical manifestations, Hurler disease causes a typical skeletal phenotype, even though its mechanism is not completely elucidated. It has been suggested that some of the skeletal abnormalities are a result of insufficient endochondral ossification, the process by which long bone is formed by replacement of cartilage. As bone and cartilage are mesenchymal lineages, we focused on the characterization of mesenchymal stem cells isolated from bone marrow (BM-MSC) of Hurler patients.
In order to explore a possible involvement of MPSI BM-MSC, our research has been focused on a novel approach which mimics in vivo the endochondral ossification process. This system is based on first forming cartilage in vitro and subsequently allowing the cartilage to be replaced by bone tissue in vivo, with the establishment of a complete bone marrow niche. IDUA mutated BM-MSC derived from MPSI patients exhibited decreased IDUA activity and increased intracellular GAG accumulation, consistent with the disease genotype. The expansion rate, phenotype, telomerase activity, and differentiation capacity towards adipocytes, osteoblasts, chondrocytes and smooth muscle cells in vitro of 3 MPSI BM-MSC lines were similar to that of BM-MSC from age matched normal control donors. MPSI BM-MSC had also similar in vivo osteogenic capacity as normal BM-MSC. However, MPSI BM-MSC displayed increased capacity to support osteoclastogenesis which may correlate with the upregulation of the RANKL/RANK/OPG molecular pathway in MPSI BM-MSC compared to normal BM-MSC. Data obtained through the in vivo transplantation of cartilage pellets differentiated from 6 MPSI BM-MSC strains have

shown in vivo a dramatic impairment in the replacement of the cartilage template by bone, suggesting a deficit in the process of endochondral ossification. The effect of IDUA deficiency on the affected endochondral bone formation process is currently under investigation by using MPSI BM-MSC strains genetically modified with an IDUA-GFP lentiviral vector. This will reveal if the insertion of the IDUA gene in MPSI BM-MSC can contribute to the reversion of the impaired mechanism. We report that BM-MSC derived from MPSIH patients can generate normal cartilage, bone and smooth muscle cells in vitro. However, MPSI BM-MSC and derived osteoblasts produce increased amounts of RANKL and display an increased capacity to stimulate the formation of osteoclasts in vitro. We have also observed a dramatic difference in the capacity of MPSI BM-MSC to recapitulate the process of endochondral ossification in vivo. Our results support the hypothesis that aberrant endochondral ossification may be one of the mechanisms involved in Hurler bone disease.

ABSTRACT N. 52

DTI - Other Genetic Diseases		
Principal Investigator	**MAFFEI MARGHERITA**	
Telethon grant N.	**TCR07007**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2008**

SKELETAL MUSCLE RESPONSE TO THE ABSENCE OF THE ANTI-OXIDANT HAPTOGLOBIN IN NORMAL CONDITIONS AND UPON METABOLIC CHALLENGE

Scabia Gaia (2,3), Bertaggia Enrico (2,4), Dalise Stefania (5), Santini Ferruccio (3), Chisari Carmelo (5), Sandri Marco (2,4), Maffei Margherita (1,2,3,6)

(1) Ospedale di Cisanello, Edificio 8, U.O. Endocrinologia, via Paradisa, 2, 56124, Pisa, Tel +39.050.995169, Fax +39.050.578778 E-mail: maffeim@immr.med.unipi.it
(2) Dulbecco Telethon Institute (DTI)
(3) Department of Clinical and Experimental Medicine, University of Pisa
(4) Venetian Institute of Molecular Medicine, Padova
(5) University-Hospital, Department of Neuroscience, Pisa
(6) CNR, Istituto Scienze Alimentazione, Pisa

Oxidative stress (OS) is considered a determinant of muscle atrophy. Despite OS known action on systems involved in protein degradation, including the ubiquitin-proteasome and the autophagy/lysosome pathways, the precise mechanisms underlying this causal/effect relationship are still debated.
Haptoglobin (Hp), an acute phase protein has important anti-oxidant function, being the main carrier of free Hb.
Aim of this study is to analyze the skeletal muscle phenotype of Hp deficient mice in normal conditions and upon metabolic challenges including physical exercise and High Fat Diet (HFD).
5 months old male Hp-/- mice reared in normal conditions show a reduction (by 10%) of cross sectional area (CSA) of tibialis anterior muscle fibers; no sign of myopathy, fiber necrosis or inflammatory infiltrate, or altered distribution of glycolytic and beta-oxidative fibers is observed. The unchanged carbonylation state of skeletal muscle proteins is accompanied by an upregulation of the antioxidant response orchestrator Nrf2. The mRNA abundance of the atrophy ubiquitin ligases, Atrogin1 and MuRF1 as well as of the autophagy related genes BNIP3 and Cathepsin L, is increased. Akt activation is downregulated, and the expression of the protein synthesis inhibitor 4EBP1 increased.
When exposed to rotarod for 3 hours, Hp-/-, but not WT mice show a significant drop in force after the exercise. Exercise induces enhanced skeletal muscle OS in both genotypes, with a more pronounced effect in Hp -/-. The induction of the mitochondriogenesis regulator PGCalpha 1, typical of physical exercise, does not take place in in Hp -/-. Cat L and Bnip3 are suppressed in both genotypes, LC3 expression is maintained at high levels in Hp-/- mice and is increased in exercised WT, with a concomitant increase of p62.
When mice are fed with HFD for 12 weeks an exacerbation of muscle atrophy was observed in Hp -/- mice (about 20%) as compared with obese WT (10%). HFD Hp-/- mice display reduced strength also in resting conditions as compared to obese WT and augmented carbonylation of skeletal muscle proteins-. Ubiquitin proteasome and autophagy systems are induced in both genotypes, but Hp-/- showed a more pronounced increase of LC3 lipidation and, as opposed to HFD WT, no increase of p62 protein.
In conclusion, Hp deficiency impacts on skeletal muscle fiber size and protein degradation prevails, despite a redox balance maintained unchanged, likely through the activation of an Nrf2 mediated antioxidant response. Under metabolic challenge that leads to increased OS, Hp-/- muscle reactivity differs from WT in that no additional antioxidant response is in place, mitochondrial biogenesis is not properly modulated, muscle performance is worsened and, upon obesity, muscle loss is exacerbated.
This study establishes the Hp -/- mouse as a useful novel model to investigate and ultimately devise strategies to block the mechanisms that from redox unbalance leads to muscle atrophy.

ABSTRACT N. 53

DTI - Other Genetic Diseases		
Principal Investigator	**RAMPOLDI LUCA**	
Telethon grant N.	**TCR08006**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2009**

CELLULAR AND ANIMAL MODELS FOR THE IDENTIFICATION OF THE PATHOGENETIC MECHANISMS IN UROMODULIN-ASSOCIATED RENAL CYSTIC DISORDERS

Schaeffer Céline (1), Trudu Matteo (1), Brunati Martina (1), Creatore Anna (1), Bernascone Ilenia (1), Amoroso Antonio (2), Ghiggeri GianMarco (3), Scolari Francesco (4), Rastaldi Maria Pia (5), Devuyst Olivier (6), Rampoldi Luca (1)

(1) DTI, Molecular Genetics of Renal Disorders, Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy, Tel. +39.02.26434947, Fax +39.02.26434767; E-mail: rampoldi.luca@hsr.it
(2) Department of Genetics, Biology and Biochemistry, University of Turin, Turin
(3) Division of Nephrology, G. Gaslini Children Hospital, Genoa
(4) Division of Nephrology, Hospital of Montichiari, Brescia
(5) Fondazione IRCCS Ospedale Maggiore Policlinico, Milan
(6) Institute of Physiology, University of Zurich, Zurich, Switzerland

Medullary Cystic Kidney Disease (MCKD) and Familial Juvenile Hyperuricemic Nephropathy (FJHN) are autosomal dominant disorders characterised by alteration of urinary concentrating ability, tubulo-interstitial fibrosis, hyperuricemia and gout, renal cysts and progressive chronic renal failure. There is no specific therapy for these disorders other than dialysis and kidney transplantation. MCKD type 2 and FJHN are caused by mutations in UMOD, the gene encoding uromodulin and are collectively referred to as uromodulin-associated kidney disease (UAKD). Uromodulin is exclusively produced in the kidney by epithelial cells of the thick ascending limb of Henle's loop and it is released in the urine where it constitutes the most abundant protein. Its role is still elusive, though it has been associated with protection against urinary tract infections and kidney stone formation, ion transport and kidney innate immunity (Rampoldi et al, 2011).
Our aim is to understand the molecular bases of pathogenesis of UAKD. To date more than 60 UMOD mutations have been reported, mostly missense changes that likely affect protein folding. We have previously demonstrated that uromodulin mutations have a common detrimental effect on protein trafficking leading to retention of mutant uromodulin in the endoplasmic reticulum (ER) (Bernascone et al, 2006). This is consistent with findings on patient biopsies showing the presence of large uromodulin aggregates in the ER of kidney epithelial cells. Moreover, we showed that mutant uromodulin can partly escape the ER quality control and reaches the plasma membrane where it aggregates with wild type protein (Schaeffer et al, 2012).
To model in vivo the effect of mutant uromodulin expression, we generated transgenic mice expressing mutant uromodulin (TgUmodC147W) (Bernascone et al, 2010). TgUmodC147W mice recapitulate most of UAKD features, developing progressive tubulo-interstitial nephritis that eventually leads to renal failure. ER retention of mutant uromodulin precedes all other features and is the primary event in the disease pathogenesis. Transcriptional profiling of kidneys from young pre-symptomatic mice showed significant upregulation of pathways related to inflammation and fibrosis. Interestingly, some key pro-inflammatory signals were already upregulated as early as at 1 week of age, well before the presence of any histological sign of renal damage.
These data clearly demonstrate a gain-of-toxic function of uromodulin mutations and provide insight into the disease pathophysiology. As uromodulin ER retention was never accompanied by induction of the unfolded protein response or apoptosis, UMOD mutations seem to exert a non cell-autonomous proteotoxic effect that is exerted by the inflammatory/fibrotic response. These findings could represent an important step towards therapeutic intervention and might have broader relevance for other tubulo-interstitial nephritis and conformational diseases.

ABSTRACT N. 54

DTI - Other Genetic Diseases		
Principal Investigator	**BOLETTA ALESSANDRA**	
Telethon grant N.	**GGP12183**	
Total budget approved €	**388.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

TOWARDS THE COMPREHENSION OF POLYCYSTIN-1 FUNCTION AND IDENTIFICATION OF SPECIFIC TARGETS FOR THERAPY IN ADPKD

Castelli Maddalena (1), Boca Manila (1), Chiaravalli Marco (1), Ramalingam Harini (2), Distefano Gianfranco (1), Carroll Thomas (2), Boletta Alessandra (1)

(1) Dulbecco Telethon Institute (DTI) at Dibit San Raffaele, via Olgettina, 58, 20132 Milano, Italy, Tel. 02.2643.4805
(2) University of Texas Southwestern Medical Center, Dallas, Texas, USA

Several organs, including lungs and kidneys, are formed by epithelial tubes whose proper morphogenesis ensures correct function. This is best exemplified by the kidney, where defective establishment or maintanance of tubular diameter results in polycystic kidney disease (PKD), a common genetic disorder. Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a slowly progressive genetic disease with an incidence of approximately 1/500-1/1000 whose main clinical manifestation is bilateral renal cyst formation. The disease results from mutation of either the PKD1 (in ~85% of cases) or PKD2 (responsible for most of the remainder) gene.
The main interest of our group is to study the normal function of Polycystin-1 (PC-1), the PKD1 gene product, in the believe that this will help elucidating the molecular basis of the disease and will explain why in the absence of PC-1 normal function renal epithelial cells degenerate and give rise to cysts. PC-1 is a large (520kDa) receptor localized at the cell-cell junctions as well as in the primary cilium in epithelial cells.
We have found that PC-1 plays an essential role in establishment of correct tubular diameter during nephron development. Recent important progress has been made in understanding the mechanisms underlying the establishment and maintenance of tubular diameter. This process is achieved by at least two mechanisms. During embryonic development both the collecting duct and the proximal tubules decrease in diameter. While cell division is randomly oriented at these stages, tubular elongation involves a process similar to convergent extension (CE) movements. It was recently shown that epithelial cells composing the tubules achieve a precise orientation and elongate mediolaterally, in a process likely resulting in cellular intercalation. This process depends on the planar cell polarity pathway (PCP) and closely resembles CE. Once this initial phase of morphogenesis is completed (approximately at postnatal day 1 in most collecting ducts in the mouse) and the optimal tubular diamater is reached, further elongation of the tubule is achieved by oriented cell division, ensuring elongation, while preserving a correct diameter. Defects in PCP altering one or both of these processes have been proposed to contribute to cystogenesis.
We have found that PC-1 is essential for proper establishment of tubular diameter and mediolateral cell orientation during embryonic renal development in the mouse. PC-1 associates with Par3 favoring the assembly of a pro-polarizing Par3/aPKC complex and it regulates a program of cell polarity important for oriented cell migration and for a convergent extension-like (CE) process during tubular morphogenesis. Par3 inactivation in the developing kidney results in defective CE and tubular morphogenesis. Our data define PC-1 as central to cell polarization and to epithelial tube morphogenesis and homeostasis.

ABSTRACT N. 55

DTI - Other Genetic Diseases		
Principal Investigator	**CORONA DAVIDE**	
Telethon grant N.	**TCR09002**	
Total budget approved €	**800.000**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2010**

AN RNA MEMORY MECHANISM TO INHERIT EPIGENETIC MARKS

Onorati Maria Cristina, Arancio Walter, Corona Davide F.V.

Dulbecco Telethon Institute c/o Università degli Studi di Palermo, Dipartimento STEMBIO, Sezione di Biologia Cellulare, Viale delle Scienze, Palermo, Tel. +39.091.23897345, E-mail: dcorona@dti.telethon.it

A central question in epigenetics is to understand how terminally differentiated daughter cells can inherit complex patterns of chromatin modifications from their mother cell. Even if several mechanisms have been hypothized to explain the establishment and maintenance of cell identity, it is still unclear how during mitosis covalent and ATP-dependent chromatin modifications are transmitted after DNA replication.
In order to unveil the molecular nature of somatic cell epigenetic memory, we used classic Position Effect Variegation assays to check if alleles of the white gene, unable to produce a main coding transcript, could modify the eye color variegation caused by an heterochromatin inversion of the white gene (wm4h). Our data show that several white alleles suppress the variegation of the wm4h line increasing eye pigmentation. Unexpectedly, the presence of nonfunctional white alleles causes an increase in the wm4h gene transcript as well as an opening in the chromatin structure at the wm4h locus. Remarkably, this effect is inheritable over several generation and is mediated by several ncRNAs encoded by the white locus, a phenomenon highly reminiscent of RNA mediated paramutation.
Our data indicate that the presence of a non-functional gene, that does not produce a coding transcript but potentially only ncRNA, could influence in trans the expression of a functional copy of the same gene silenced by heterochromatin. Our data strongly suggest that cells can 'sense' the presence of non coding RNA's inherited from their mother cells and can use them to epigenetically reset their transcriptional program after DNA replication.

TELETHON RESEARCH PROJECTS

ABSTRACT N. 56

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MINCHIOTTI GABRIELLA**	
Telethon grant N.	**GGP08120**	
Total budget €	**261.800**	
Centres: **1**	*Duration (yrs):* **5**	*Starting year:* **2008**

A NOVEL ROLE OF THE EGF-CFC CRIPTO IN MUSCLE REGENERATION AND DISEASE

Guardiola Ombretta (1), Iaconis Salvatore (1), Andolfi Gennaro (1), Tajbakhsh Shahragim (2), Minchiotti Gabriella (1)

(1) Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Via Pietro Castellino 111, Naples, Italy, Tel. 0816132357, E-mail: gabriella.minchiotti@igb.cnr.it
(2) Institut Pasteur, Stem Cells & Development, CNRS URA 2578, Paris, France

Skeletal muscle tissue responses following injury and disease are highly complex and coordinated processes involving the interactions of many different cell populations that promote inflammation, muscle regeneration, and angiogenesis, whose knowledge is still limited. Thus a better understanding of the molecular and cellular interactions of these complex events is crucial to promote successful therapies for diseases associated with muscle wasting such as dystrophynopathies. We have identified the developmental protein Cripto as a critical regulatory molecule in this complex scenario, and a promising therapeutic target. Cripto is an extracellular protein essential for early vertebrate development, and a hallmark of pluripotent embryonic stem cells, however its role in adult life still remains elusive. We have recently shown that Cripto is re-expressed in skeletal muscle in response to injury and that this is the basis for an efficient regeneration. Both myogenic and inflammatory cells express Cripto during skeletal muscle regeneration. Thus to evaluate the cellular contribution of Cripto in muscle regeneration, we have generated a novel conditional satellite cell –specific cripto knock out mice, and demonstrated that inactivation of cripto in adult satellite cells compromises skeletal muscle regeneration, thus providing direct evidence that cripto is required in satellite cells for effective regeneration. Moreover, we demonstrated that soluble Cripto (sCripto) protein fully rescued muscle regeneration defects, thus providing direct evidence that sCripto was able to fully recapitulate the function of endogenous membrane Cripto. All together our data provide direct evidence for a major role for Cripto in satellite cells and in the complex process of skeletal muscle regeneration, and open the way to assess the potential of Cripto protein in the treatment of muscle disorders.

ABSTRACT N. 57

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CARUSO MAURIZIA**	
Telethon grant N.	**GGP08126**	
Total budget €	**199.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

ROLE OF CYCLIN D3 IN SATELLITE CELL FUNCTION AND MUSCLE REGENERATION

De Luca Giulia, Ferretti Roberta, Bruschi Marco, Caruso Maurizia

CNR, Istituto di Biologia Cellulare e Neurobiologia, Via del Fosso di Fiorano 64, 00143 Roma, Tel. 06 501703186, Fax 06 501703311, E-mail: maurizia.caruso@cnr.it

The regenerative potential of adult muscle depends on satellite cells: specialized stem cells resident beneath the basal lamina of mature muscle fibres. A thorough understanding of the regulatory mechanisms controlling the function of these cells is essential to devise new strategies for regeneration in muscle dystrophy. In healthy muscle, satellite cells are mitotically quiescent, but in response to muscle damage or degenerative disease, they reenter the cell cycle and, after several rounds of cell divisions, either differentiate forming new myofibers or self-renew to replenish the stem cell pool.
We have previously shown that cyclin D3 is induced during myoblast differentiation in vitro through mechanisms controlled by MyoD and pRb, two pivotal regulators of skeletal myogenesis (Cenciarelli et al. Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. Mol. Cell. Biol. 1999, 19, 5203-5217. De Santa et al. pRb-Dependent Cyclin D3 Protein Stabilization Is Required for Myogenic Differentiation. Mol. Cell. Biol. 2007, 27, 7248-7265). In this study, we defined the role of cyclin D3 in the control of satellite cell function by using RNAi technology to knockdown cyclin D3 protein levels in vitro and cyclin D3 knockout approach in vivo.
The inhibition of cyclin D3 expression in satellite cell-derived C2 myoblasts resulted in reduced proliferation, premature expression of differentiation markers and impaired myotube formation, indicating that cyclin D3 critically controls the balance between myoblast proliferation and differentiation.
Cyclin D3 knockout mice displayed no evident muscle defect or pathology, although they presented smaller body size and muscle mass compared with wild-type littermates. Cyclin D3-null adult muscle showed fewer quiescent satellite cells and smaller myofibres than wild-type muscle, which suggested that cyclin D3 is involved in post-natal muscle growth by controlling satellite cell function. The analysis of primary myoblast and of satellite cells associated to single myofibres in culture indicated that cyclinD3-null myogenic progenitors have a reduced proliferative potential, a higher propensity to differentiate and a reduced ability to self-renew compared to wild type controls.
Consistently with the effects observed in vitro, cyclin D3-null myogenic progenitors in the regenerating muscle displayed a significantly decreased proliferation rate and premature induction of myogenic differentiation compared to wild-type. Furthermore, at the end of regeneration, cyclin D3-null muscle showed a decrease in regenerated myofiber size and a reduced number of cells repopulating the satellite cell niche.
Our results demonstrate that cyclin D3 is critically required during muscle regeneration for proliferative expansion and self-renewal activity of satellite cells.

ABSTRACT N. 58

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SALVATORE DOMENICO**	
Telethon grant N.	**GGP11185**	
Total budget €	**213.900**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE INTRACELLULAR CONTROL OF THYROID HORMONE SIGNALING IN MUSCLE STEM CELLS AND IN DUCHENNE MUSCULAR DYSTROPHY

Dentice Monica, Ambrosio Raffaele, Sibilio Annarita, Luongo Cristina, Salvatore Domenico

Department of Molecular and Clinical Endocrinology and Oncology, University of Naples "Federico II" - Tel. +39 081 7463780, Fax +39 081 7463668; E-mail: domsalva@unina.it

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, causing a severe, progressive muscle wasting. The active thyroid hormone (T3) derives either directly from thyroid secretion or by the deiodination of the prohormone thyroxine (T4). Much of the intracellular T3 derives from D2, the only activating-deiodinase expressed in muscle, while the deiodinase (D3) has an opposing function, catalyzing the inactivation of T4 and T3. Our previous studies clearly indicated that D2 is an essential component of the myogenic program in muscle development and regeneration. Dio2 null muscles have a reduced intracellular T3 and significant myogenic and regenerative defects despite normal concentrations of circulating thyroid hormone (TH).
The general objective of this study is to dissect the role of TH signaling and its local control by deiodinases in muscle stem cell biology and in the regeneration process. By using genetically-manipulated mouse lines and primary culture of stem cells, we aim to use the intracellular manipulation of thyroid hormone concentrations to modify the physiology of muscle stem cell.
Our preliminary data strongly suggest that the deiodinases control of TH action significantly affects the physiology of muscle stem cell. Most importantly, these changes are reversible, as the intracellular hypothyroidism caused by D2-depletion can be reversed by appropriate T3 replacement, opening the possibility to use deiodinase as a novel therapeutic target for muscle stem cells. We report here that, in response to proliferative stimuli such as skeletal muscle acute injury, type 3 deiodinase (D3) - the thyroid hormone-inactivating enzyme - is specifically induced in satellite cells, where it reduces intracellular thyroid signaling. Satellite-specific genetic ablation of Dio3 impairs skeletal muscle regeneration.
In conclusion, our results indicate that the D3 enzyme is dynamically exploited in vivo to attenuate TH-signaling and simultaneously orchestrate distinct gene activation and repression programs required for the satellite cell lineage progression and survival.
Dentice M, Marsili A, Ambrosio R, Guardiola O, Sibilio A, Paik JH, Minchiotti G, DePinho RA, Fenzi G, Larsen PR, Salvatore D. The FoxO3/type 2 deiodinase pathway is required for normal mouse myogenesis and muscle regeneration. J Clin Investigations. 2010 Nov 1;120(11):4021-30
Marsili A, Ramadan W, Harney JW, Mulcahey M, Castroneves LA, Goemann IM, Wajner SM, Huang SA, Zavacki AM, Maia AL, Dentice M, Salvatore D, Silva JE, Larsen PR. Type 2 Iodothyronine Deiodinase Levels Are Higher in Slow-Twitch than Fast-Twitch Mouse Skeletal Muscle and Are Increased in Hypothyroidism. Endocrinology. 2010 Dec;151(12) : 5952-60.

ABSTRACT N. 59

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CLEMENTI EMILIO**	
Telethon grant N.	**GGP07006**	
Total budget €	**268.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2007**

MOLECULAR BASIS OF THE THERAPEUTIC EFFECT OF NITRIC OXIDE AND THE NITRIC OXIDE-RELEASING ANTI-INFLAMMATORY DRUG NITROFLURBIPROFEN IN MUSCULAR DYSTROPHY: ANALYSIS OF MITOCHONDRIAL STRUCTURE AND FUNCTION IN DEVELOPING AND REGENERATING SKELETAL MUSCLE

De Palma Clara (1), Touvier Thierry (2), Brunelli Silvia (3), Clementi Emilio (1)

(1) Università degli Studi di Milano - Dip Scienze Biomediche e Cliniche L. Sacco, via GB Grassi 74, 20157 Milano, E-mail: emilio.clementi@unimi.it; Tel. 3666227327, 0250319646
(2) IRCCS E. Medea, Bosisio Parini, LC
(3) Dip Medicina Sperimentale, Università di Milano Bicocca, Milano

The therapy of muscular dystrophy still relies on administration of corticosteroid, which is not resolutive and accompanied by severe side effects. The stem cell and gene therapy approaches currently tested may be useful only for specific subsets of patients and are extremely expensive. Pharmacological treatments, while not resolving the genetic defect, may still offer significant advantages as they may be useful to all patients, at affordable costs, and may delay disease progression. This project is designed to discover novel therapeutic targets and assess their validity in the mdx mouse model of dystrophy. During this project, now terminated, we have demon-

strated in preclinical studies the efficacy of nitric oxide (NO)-donating drugs when associated with non steroidal anti inflammatory drugs in slowing disease progression. We have also investigated the mechanisms whereby NO is effective and identified in the control of mitochondrial dynamics and bioenergetics significant aspects. In particular we identified in the mitochondrial fission-inducing protein DRP-1 a key molecule, as part of the reparative myogenic programme fostered by NO is mediated through inhibition of this protein. We have generated a mouse model over expressing DRP-1 in a muscle specific way. This model recapitulates key functional and structural alterations found in human myopathies.

ABSTRACT N. 60

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**COSSU GIULIO**	
Telethon grant N.	**GGP08030**	
Total budget €	**329.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

REVERSIBLE IMMORTALIZATION AND TRANSDUCTION WITH A DYSTROPHIN ARTIFICIAL CHROMOSOME OF HUMAN DMD MESOANGIOBLASTS FOR THE CELL THERAPY OF MUSCULAR DYSTROPHY

Benedetti Sara (1), Tedesco Francesco Saverio (1), Hoshiya Hidetoshi (1), D'Antona Giuseppe (2), Gerli Mattia (1), Ragazzi Martina (1), Yasushiro Kazuki (3), Tonlorenzi Rossana (1), Chaouch Soraya (4), Lombardo Angelo (5), Antonini Stefania (6), Gargioli Cesare (7), Naldini Luigi (5,1), Torrente Yvan (8), Bottinelli Roberto (2), Messina Graziella (6), Oshimura Mitsuo (3), Cossu Giulio (1,6)

(1) Division of Regenerative Medicine San Raffaele Hospital. Via Olgettina 58 20132 Milan, Fax +390226434621, E-mail: cossu.giulio@hsr.i
(2) Department of Physiology and Interuniversity Institute of Myology, University of Pavia, 27100, Pavia
(3) Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Tottori University, Yonago, Japan
(4) Universitè Pierre et Marie Curie/Paris 6/Inserm UMR_S 974, CNRS UMR 7215, Institut de Myologie, Groupe Hospitalier Pitie-Salpetriere, Paris
(5) San Raffaele Telethon Institute for Gene Therapy, 20132 Milan, Italy
(6) Department of Life Sciences, University of Milan, 20133, Milan, Italy
(7) Departmentof Biology, University of Tor Vergata, Rome
(8) Stem Cell Laboratory, Department of Neurological Science, University of Milan

Duchenne Muscular Dystrophy (DMD) is a genetic disorder characterized by the lack of the dystrophin protein that leads to progressive wasting of skeletal muscle. This project aims to develop a new cell therapy approach to treat DMD based on the transplantation of dystrophic mesoangioblasts (MABs, vessel-associated stem cells), genetically corrected with a Human Artificial Chromosome (HAC) containing the entire 2.4Mb human dystrophin locus (DYS-HAC). HACs have many advantages over conventional gene therapy vectors, such as episomal maintenance and the ability to carry large genetic regions with regulatory elements: however, there is currently no evidence of efficacious therapy using HACs in any genetic disorder. We proved the feasibility of this strategy by transferring the DYS-HAC in MABs derived from the DMD mouse model mdx (mdx(DYS-HAC)MABs), that are spontaneously immortal. DYS-HAC expressing mdx(DYS-HAC)MABs robustly engrafted skeletal muscle and produced many dystrophin positive fibers in dystrophic mice, leading to a significant morphological and functional amelioration that lasted for most of the mouse lifespan (Tedesco et al. Sci. Transl. Med. 2011). However, in order to extend this strategy to human MABs (hMABs), an additional step of immortalization is required, since hMABs would undergo replicative senescence during the selection phase needed to isolate cells where the DYS-HAC has been transferred. In order to reversibly immortalize both normal and dystrophic hMABs, we used lentiviral vectors encoding floxed IRES-HSV1-TK hTERT, the catalytic subunit of telomerase and floxed IRES-HSV1-TK polycomb group gene Bmi-1 respectively. Three normal clones have been characterized for their proliferative ability and proper expression of hTERT and Bmi-1. Moreover, despite the immortalization process, the clones remain growth factor dependent, contact inhibited, not tumorigenic and still able to undergo in vitro myogenic differentiation. We then immortalized hMABs from DMD patients and transferred of a new generation DYS-HAC, devoid of immunogenic transgenes such as GFP (DYS-HAC2). Up to now, we obtained three DMD hMAB immortalized clones containing the DYS-HAC2 as revealed by both by PCR and FISH analysis. Additionally, we are now testing the reversibility of the system through the use of a non-integrating lentiviral vector expressing Cre recombinase and the in vivo ability of DYS-HAC2 immortalized DMD hMAB clones to engraft dystrophic skeletal muscle and differentiate into dystrophin expressing myofibers.

ABSTRACT N. 61

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**COSSU GIULIO**	
Telethon grant N.	**GSP11002**	
Total budget €	**716.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2011**

CELL THERAPY OF DUCHENNE MUSCULAR DYSTROPHY BY INTRA-ARTERIAL DELIVERY OF HLA-IDENTICAL ALLOGENEIC MESOANGIOBLASTS

Cossu Giulio (1), Napolitano Sara (2), Cicalese Maria Pia (2), Previtali Stefano (3), Venturini Massimo (4), Politi Letterio Salvatore (5), Fiori Rossana (6), Tedesco Francesco Saverio (1), Noviello Maddalena (1), Tettamanti Andrea (7), Nicastro Francesca (7), Godi Claudia (7), Marktel Sarah (2), Natali-Sora Maria Grazia (3), Scarlato Marina (3), Ciotti Francesca (2), Giuliani Serena (2), Tonlorenzi Rossana (3), Bonini Chiara (1), Torrente Yvan (8), Ciceri Fabio (2)

(1) Division of Regenerative Medicine San Raffaele Hospital Via Olgettina 58 20132 Milan, Fax +39.02.26434621, E-mail: cossu.giulio@hsr.it
(2) Hematology and BMT Unit, Department of Oncology, San Raffaele Hospital, Milano, Italy
(3) Institute of Experimental Neurology, San Raffaele Scientific Hospital, Milan, Italy
(4) Department of Radiology, San Raffaele Hospital, Milan,
(5) Department of Neuroradiology, San Raffaele Hospital, Milan
(6) Department of Anesthesiology, San Raffaele Hospital, Milan
(7) Physiotherapy and Rehabilitation, San Raffaele Hospital, Milan
(8) Department of Neurology and Laboratory of Neuroscience, University of Milan, Ospedale Maggiore Policlinico, Milan

A mono-centric, sequential, non-randomized, open-label clinical phase I-II study of cell therapy based upon intra-arterial transplantation of allogeneic human mesoangioblasts from an HLA-identical family donor is being carried out in five pediatric patients affected by Duchenne Muscular Dystrophy under immunosuppressive treatment with Tacronimus. The protocol foresees four consecutive injections (at two months intervals), at escalating doses of cells.
The primary objectives of this study are to assess the incidence of adverse events and to determine the effect of multiple intra-arterial injections in modifying muscle strength in DMD patients.
Mesoangioblasts are vessel-associated progenitors that can be expanded in vitro and have shown efficacy in ameliorating structure and function of dystrophic muscle in murine and canine models of muscular dystrophy.
Human cells have been characterized and expanded as medicinal product under GMP conditions at MolMed, Milan.
Toxicology studies carried out at Accelera, Nerviano, have shown absence of acute and chronic toxic effects. Finally, a 1 year observational study (DMD01) has been completed on 28 DMD patients in order to test their muscle strength at 3 months intervals (Lerario et al. BMC Neurology 2012): 5 of these patients are eligible for the transplantation trial because of an HLA-identical donor. The results of the first three patients indicated a stabilization of motor and contractile activity that was deteriorating before cell transplantation, in two out of three patients. The third patient was already wheel chair confined 6 months before start of the trial. In addition we found donor DNA (up to 0.7%) in two patients and donor dystrophin by Western Blot analysis in one. This patient has no T cells reactive towards donor cells. Two more patients are starting transplantation in November 2012 and the results will be available towards the end of 2013.

ABSTRACT N. 62

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FERLINI ALESSANDRA**	
Telethon grant N.	**GGP09093**	
Total budget €	**515.700**	
Centres: **4**	*Duration (yrs):* **2**	*Starting year:* **2010**

PRE-CLINICAL EVALUATION OF BIOCOMPATIBLE NANOPARTICLES AS DELIVERY SYSTEM OF 20-METHYL-PHOSPHOROTHIOATE (2OMEPS) ANTISENSE OLIGORIBONUCLEOTIDES FOR EXON SKIPPING-MEDIATED DYSTROPHIN IN RESTORATION

Falzarano Maria Sofia (1), Bassi Elena (1), Fabris Marina (1), Perrone Daniela (2), Sabatelli Patrizia (3), Maraldi Nadir (3), Dona' Silvia (4), Bonaldo Paolo (4), Braghetta Paola (4), Rimessi Paola (1), Sparnacci Katia (5), Laus Michele (5), Ferlini Alessandra (1)

(1) Department of Medical Sciences, Section of Medical Genetics, University of Ferrara, Italy, Via Fossato di Mortara, 74, 44100 Ferrara, Tel 0532.974439, Fax 0532.974406, E-mail: fla@unife.it
(2) Department of Biology and Evolution, University of Ferrara, Ferrara, Italy
(3) IGM-CNR, Unit of Bologna c/o IOR, Bologna, Italy
(4) Department of Biomedical Sciences, University of Padua, Padua, Italy
(5) Department of Environmental and Life Sciences INSTM, University of Eastern Piedmont, Alessandria, Italy

We have previously demonstrated that intraperitoneally (i.p.) injections of 2'-O-methyl-phosphorothioate antisense oligoribonucleotides (AONs) loaded onto ZM2 nanoparticles (NPs) induce dystrophin restoration in the mdx mice muscles (Ferlini et al. 2010 Gene Ther; Bassi et al. 2012 J Biom Biotech). In this work we studied: i) pharmacodynamic and pharmacokinetics of ZM2 by testing the NPs biodistribution and clearance both in tissues and in fluids (urine, feces) after i.p. or oral administrations; ii) the oral route as an alternative way of administration for ZM2-AON complexes; iii) a novel smaller NPs (ZM5) to improve intestinal adsorption and biodistribution by oral administration. To evaluate the biodistribution and elimination of ZM2 we used the Odyssey ® Infrared Imaging System (Li-Cor); we modified ZM2 cationic surface to allow the binding with IRDye (ZM4-IR) and to evaluate biodistribution and elimination (faeces and urine anlysis) in i.p. or orally administered mdx mice. After in vivo imaging, excised tissues were imaged ex vivo to confirm and quantify accumulation of the NPs in tissues and organs.
After single i.p. injection, ZM4-IR fluorescence is visible widely in all the body, especially in lymphatic tissues suggesting a body distribution via lymphatic vessels. A single oral administration of ZM4-IR demonstrates the presence of NPs in intestinal lumen for at least 48 hours. Odyssey analysis of muscle cryosections from mice treated with multiple ZM5-IR injections shows fluorescence until 60 days after the last inoculation. In orally treated mdx mice, only the ileum results fluorescent at 7 days after last administration and all tissues analysed at 30 days after last treatment, no fluorescent signal is detectable even in gut sections. In i.p. route, NPs are present both in the feces and urine even after 3 weeks; in oral route ZM4-IR are mainly cleared in the feces.
We subsequently tested alginate-free and alginate-encapsulated ZM2-AON complexes orally administered in mdx mice for 12 weeks. We observe a moderate rescue of dystrophin protein in the intestinal smooth muscles and a mild positivity in the diaphragm only when in the presence of alginate. Moreover, we synthetized and tested a new NP sample (ZM5), smaller in size and not containing the hydrophilic comonomer NIPAM, at variance from ZM2 NPs. The Odyssey in vivo imaging time course of ZM5-IR single or multiple administration demonstrates the persistence of ZM5-IR in intestinal lumen for 48 hours. Odyssey analysis of cryosections shows ZM5-IR in the intestine, liver and diaphragm. Clearance studies demonstrate that the majority of administered ZM5-IR are eliminated, as well as ZM4-IR, almost exclusively through feces in the first 36 hours. Preliminary results show that ZM5 overcomes the intestinal barrier with a wide biodistribution into the body muscles. These results encourage further research toward the appealing oral administration route for antisense molecules.

ABSTRACT N. 63

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**TORRENTE YVAN**	
Telethon grant N.	**GGP09292**	
Total budget €	**98.100**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2009**

CELL THERAPY OF MUSCULAR DYSTROPHY WITH ENGINEERED CD133+ STEM CELLS

Meregalli Mirella (1), Farini Andrea (1), Belicchi Marzia (1), Parolini Daniele (1), Sitzia Clementina (1), Razini Paola (1), Cassinelli Letizia (1), Da Silva Bzario Joao Carlos (2), Garcia Luis (3), Torrente Yvan (1)

(1) Laboratorio Cellule Staminali, Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico di Milano, Centro Dino Ferrari, via F. Sforza, 35 20122 Milano, Tel. 0039-02-55033874, Fax 0039-02-50320430; E-mail: yvan.torrente@unimi.it
(2) AADM/UNAERP Ribeirao Preto-SP-Brazil
(3) UMR S 787, INSERM/UPMC, Institut de Myologie, Facultè de Medecine Pierre et Marie Curie, Paris Cedex 13, France

In DMD, skeletal and cardiac muscles are affected. Cell therapy is one promising approach to correct genetic diseases by contributing to tissue regeneration; stem cells can be isolated from a healthy donor or, when possible from the same patient. In the first case cells will be transplanted under a regime of immune suppression while in the second case, cells will have to be genetically corrected before transplantation in the same patient from which they were derived. We investigated exon skipping approach in order to find new tools for the treatment of DMD. We treated GRMD dogs, the most reliable animal model that shows a form of dystrophy very similar to DMD. We isolated CD133+ cells from muscle biopsies of GRMD dogs one year old characterized by a mild and severe clinical phenotype in order to demonstrate the efficacy of the treatment in old dogs and after in old patients. Dogs were treated with their own transduced U7exon 6-8 CD133+ cells. All dogs received 2 arterial systemic injections. After the injection, we performed different functional measures in order to test whether morphological and biochemical changes: claim Stairs (Time), swimming, 6 minute walking test (6MWT). Dogs were followed for two years after the injection; dogs not injected or injected with not engineered CD133+ stem cells died in one year. Dogs transplanted with engineered CD133+ stem cells survived for two years after the injection; we showed that the autologous transplantation increased the survival of the dogs. Dystrophin expression in the biopsies was variable, ranging from 2 to = 7% in biopsies of the injected legs and detectable after one year from the first injection. Western blot analysis of muscle biopsies confirmed the presence of different amount of dystrophin, varying from an undetectable signal to around 6% of a wt canine muscle. The pre-clinical validation of this approach leads to a new treatment of muscular dystrophies based on the combination of gene and stem cell therapy. There are different mutations that cause DMD and most of them can be target with methods that have been developed for gene correction. This mutation-focused approach offers the opportunity for personalized gene and stem cell treatment for muscular dystrophies and might also be a logical strategy for the treatment of other genetic disorders.

ABSTRACT N. 64

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MATTEI ELISABETTA**	
Telethon grant N.	**GGP10094**	
Total budget €	**124.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

EXPERIMENTAL GENE THERAPY OF DUCHENNE MUSCULAR DYSTROPHY BY ARTIFICIAL TRANSCRIPTION FACTORS UP-REGULATING THE DYSTROPHIN-RELATED GENE UTROPHIN

Passananti Claudio (2), Strimpakos Georgios (1), Onori Annalisa (2), Pisani Cinzia (2), Di Certo Maria Grazia (1), Severini Cinzia (1), Luvisetto Siro (1), Monaco Lucia (3), Corbi Nicoletta (2), Mattei Elisabetta (1)

(1) Istituto Biologia Cellulare e Neurobiologia, CNR, Via del Fosso di Fiorano 64, 00143, Roma, Tel. +39.06.50170323, Fax +39.06.501703313, E-mail: elisabetta.mattei@inmm.cnr.it
(2) Istituto di Biologia e Patologia Molecolari, CNR, Roma
(3) Dipartimento di Fisiologia e Farmacologia, Università La Sapienza, Roma

The cure of Duchenne Muscular Dystrophy (DMD) depends on the development of innovative therapies able to improve the quality of life and to extend the survival of DMD patients. Over-expression of the dystrophin-related genes utrophin represents a promising therapeutic approach. To achieve utrophin up-regulation, we engineered artificial, zinc finger based, transcription factors (ZF ATFs) capable of binding and activating transcription from the promoter "A" of both human and mouse utrophin genes.
Using the dystrophin-deficient mdx mice model, we generated a transgenic mdx mice expressing the artificial transcription factor mini-gene named "Jazz". In mdx-Jazz mice model Jazz protein is able to specifically up-regulate the target gene utrophin at muscular level and it induces a significant recovery of muscle strength.

Here we demonstrate the feasibility and efficacy of the systemic delivery of our Jazz mini gene in mdx mice by recombinant adeno-associated-virus vectors (rAAVs). In particular, to achieve Jazz muscle specific expression, we used the combination of muscle specific alpha-Actin human promoter and the AAV8 serotype with high muscle tropism. Furthermore, to easily follow viral infection, we constructed a second molecule substituting Jazz with green fluorescent protein (GFP). Neonatal mdx mice were intraperitoneally injected with AAV8-Jazz or with AAV8-GFP viruses. Both viruses showed very similar systemic diffusion and transduction efficiencies, characterized by an early as well as strong expression in skeletal and cardiac muscles. The analysis of typical diagnostic criteria for DMD showed that AAV8-Jazz improves muscle pathology as compared to non-treated or AAV8-GFP infected mdx mice. Moreover, performing treadmill tests on AAV8-Jazz infected mdx mice and electrophysiological tests on isolated muscles, we provided evidences for functional muscle recovery. These results confirm the feasibility and efficacy of ZF ATF strategy in gene therapy and accelerate the clinical translation process for a possible "Jazz- therapy" in human Duchenne muscular dystrophy.

ABSTRACT N. 65

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BOZZONI IRENE**	
Telethon grant N.	**GGP11149**	
Total budget €	**305.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

RNA-BASED GENE THERAPY OF DUCHENNE MUSCULAR DYSTROPHY: ROLE OF miRNA DEREGULATION IN THE PATHOGENESIS OF DMD AND THEIR POSSIBLE USE FOR IMPROVING THE EXON SKIPPING STRATEGY

Cazzella Valentina, Morlando Mariangela, Legnini Ivano, Martone Julie, Majello Sara, Briganti Francesca, Bozzoni Irene

Dept. of Biology and Biotechnology, Sapienza University of Rome, Tel. 06-49912202, E-mail: irene.bozzoni@uniroma1.it

One of the greatest surprises of high throughput transcriptome analysis of the last years has been the discovery that the mammalian genome is pervasively transcribed into many different complex families of RNA. It is now becoming largely accepted that the non-coding portion of the genome rather than its coding counterpart is likely to account for the greater complexity of higher eukaryotes. In addition to a large number of alternative transcriptional start sites, termination and splicing patterns, a complex collection of new antisense, intronic and intergenic transcripts was found.
Small non-coding RNA have been extensively studied and shown to be key components of regulation and control in many eukaryotes including animals and plants. While they were initially described as negative switches acting with transcription factors to control of gene expression, these RNAs are now seen as modulators or fine tuners of posttranscriptional regulation that are often components of negative or positive feedback loops.
Although many studies have helped unveiling the function of many small non-coding RNAs, very little is known about the long non-coding (lncRNA) counterpart of the transcriptome.
Examples of regulatory circuitries regulated by both small and long non-coding RNAs will be presented and perspectives on their new functions in the control of gene expression in normal and Duchenne muscles will be discussed.

ABSTRACT N. 66

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MESSINA SONIA**	
Telethon grant N.	**GUP09010**	
Total budget €	**189.000**	
Centres: **10**	*Duration (yrs):* **2**	*Starting year:* **2010**

OUTCOME MEASURES IN DUCHENNE MUSCULAR DYSTROPHY: VALIDATION OF THE PEDIATRIC QUALITY OF LIFE INVENTORY TM NEUROMUSCULAR MODULE IN THE ITALIAN POPULATION AND CORRELATION WITH OTHER FUNCTIONAL ASSESSMENTS

Mercuri Eugenio (10), Vita Giuseppe (1), Bertini Enrico (2), Berardinelli Angela (3), Minetti Carlo (4), Politano Luisa (5), Mongini Tiziana (6), Pegoraro Elena (7), Morandi Lucia (8), D'Angelo Maria Grazia (9), Messina Sonia (1, 10)

(1) Dipartimento di Neuroscienze, Università di Messina, Via C. Valeria 1, 98125, Messina, Tel. 090.2212793, Fax 090.2212789, E-mail: smessina@unime.it
(2) Unità Malattie Neuromuscolari, Ospedale Bambin Gesu', Roma
(3) Unità di Neuropsichiatria Infantile, Istituto IRCCS "C.Mondino", Pavia
(4) Istituto IRCCS "Gaslini", Genova
(5) Dipartimento di Medicina Sperimentale, Seconda Università, Napoli
(6) Centro per le Malattie Neuromuscolari "Peirolo", Dipartimento di Neuroscienze, Università di Torino
(7) Dipartimento Di Neuroscienze, Università di Padova, Padova
(8) Istituto Neurologico IRCCS "C. Besta", Milano
(9) IRCCS Medea Bosisio Parini
(10) Dipartimento di Pediatria, Università Cattolica del Sacro Cuore, Policlinico Gemelli, Roma

Objectives: the main aim of the study was to translate and validate the Pediatric Quality of Life Inventory TM 3.0 Neuromuscular Module (PedsQL NM) in combination with other selected outcome measures, PedsQL TM 4.0 Generic Core Scales (PedsQL GCS), 6-minute walk test (6MWT), North Star Ambulatory Assessment (NSAA) and timed items, in a large cohort of Italian patients with Duchenne muscular dystrophy (DMD). We also aimed to collect longitudinal data over 6 and 12 month interval of all the measurements and to verify possible correlations among them.
Background/Rationale: Main issues in designing multicentric clinical trials is the lack of validated instruments to assess quality of life (QoL) in children with neuromuscular disorders (NMD) and the lack of an international agreement on the outcome measures to be used. Recently the PedsQL NM has been proposed as a reliable tool to assess QoL in children with NMD. The questionnaire, available as parents' and patient's version, has been validated in spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD). However so far no systematic study has been planned to correlate QoL results with the outputs of the "gold standard" outcome measures for DMD.
The project is ongoing. We performed the translation of the PedsQL NM in Italian and training sessions of the protocol administration (PedsQLNM and GCS and assessment with 6MWT, NSAA and timed items). We enrolled 157 DMD patients (age range 2,4-17,8 yrs), 107/157 patients were ambulant and therefore correlations with the selected outcome measures were possible. We performed assessment at baseline and 6 and 12 months thereafter. Analysis of the data are in progress. Anticipated output: validate for the first time a QoL tool specific for the DMD population in correlation with outcome measures available for clinical trials and collect longitudinal information on the changes observed over 1 year period.

ABSTRACT N. 67

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PANE MARIKA**	
Telethon grant N.	**GUP11002**	
Total budget €	**300.000**	
Centres: **12**	*Duration (yrs):* **3**	*Starting year:* **2012**

ASSESSMENT OF UPPER LIMB FUNCTION IN NON AMBULANT DUCHENNE MUSCULAR DYSTROPHY

Pane Marika (1), Mazzone Elena (1), Berardinelli Angela Lucia (2), Messina Sonia (3), D'Amico Adele (4), Baranello Giovanni (5), Gardani Alice (2), Capone Luca (2), Vita Gianluca (3), Cavallaro Filippo (3), Catteruccia Michela (4), Colia Giulia (4), Iotti Elena (5), D'Ambrosio Paola (6), Taglia Antonella (6), Rolle Enrica (7), Lanzillotta Valentina (8), Pini Antonella (9), Terzi Silvia (9), Talarico Maria Laura (9), Frosini Silvia (10), D'Angelo Maria Grazia (11), Molteni Francesca (11), Fiorillo Chiara (10), Battini Roberta (10), Bello Luca (12), Cao Michelangelo (12), Mongini Tiziana (7), Pedemonte Marina (8), Pegoraro Elena (12), Morandi Lucia (5), Politano Luisa (6), Mercuri Eugenio (1)

(1) Department of Paediatric Neurology, Catholic University, Policlinico Gemelli, Largo A. Gemelli, 8, 00168, Rome, Italy 347/9347864 E-mail: marika.pane@rm.unicatt.it
(2) Child Neurology and Psychiatry Unit, IRCCS "C. Mondino" Foundation, University of Pavia, Italy
(3) Department of Neurosciences, Psychiatry and Anaesthesiology, University of Messina, Messina, Italy
(4) Department of Laboratory Medicine, Unit of Molecular Medicine, Bambino Gesù Hospital, Rome, Italy

(5) Myopathology and Neuroimmunolgy, Pediatric Neurology and Neuroradiology Units, Neurological Institute C. Besta, Milan, Italy
(6) Department of Experimental Medicine, Second University of Naples, Italy
(7) Neuromuscular Center, SG. Battista Hospital, University of Turin, Italy
(8) Neuromuscular Disease Unit, G. Gaslini Institute, Genoa, Italy
(9) Child Neurology and Psychiatry Unit, Maggiore Hospital, Bologna, Italy
(10) Department of Developmental Neuroscience, Stella Maris Institute, University of Pisa, Italy
(11) IRCCS Eugenio Medea, Bosisio Parini, Italy
(12) Department of Neurosciences, University of Padua, Padua, Italy

While there have been considerable advances on outcome measures for ambulant DMD boys, less has been reported on possible measures in non ambulant older boys and adults, with increasing concern from families and patient groups. This information appears to be highly important not only for a better understanding of disease progression but also to allow a possible enrolment of these patients in future trials.
The aim of the project is to collect data on a large number of patients to assess the suitability of different measures assessing upper limb function. As DMD is a relatively rare condition, such goal can only be reached in a multicentric setting. Each centre will therefore contribute on average with approximately 15 to 20 patients. All the participating centres (Rome Gemelli, Messina, Rome Bambin Gesù, Pavia, Genoa, Naples, Turin, Bologna, Padua, Milan, Bosisio Parini, Pisa) have a good record of cooperation in similar projects having already been involved in the validation of the North Star in DMD and of the Hammersmith Functional Motor Scale for SMA.
Each centre will recruit and evaluate DMD patients. In the first months of the project we have already achieved the following goals following this schedule:
1. Preliminary discussion of the possible measures to be used in the project and choice of a meaures (Performance Upper Limb, PUL) recently developed by an international group.
2. Training and interobserver reliability sessions.
After a preliminary meeting involving both clinicians and physiotherapists, two meeting have been organized to train the physiotherapists from each participating center in the administration of the PUL scale with hands-on practice and video scoring. A second meeting helped to rediscuss the scale after some practice and establish intra-rater and inter-rater reliability on the used outcome measures through video scoring.
3. Enrollment of the patients fulfilling the inclusion criteria.
In the second part of the project. once enrolment is completed, patients will be followed at 6, 12 and 24 month intervals. Data will be entered on a CRF and on a database and the databases will be sent monthly to the coordinating centre. The dedicated research fellow of the Coordinator Centre will travel among centres and collect all the CRF forms and check the data entered in the database. After baseline the dedicated physiotherapist of the Coordinator Centre will travel among centers to monitor the administration procedures that will be kept in agreement with the training. The dedicated research fellow and the statistical advisor will collaborate to the analysis. A final meeting will be organized to discuss the results.

ABSTRACT N. 68

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MAGLIANO LORENZA**	
Telethon grant N.	**GUP10002**	
Total budget €	**145.000**	
Centres: **9**	*Duration (yrs):* **2**	*Starting year:* **2011**

THE FAMILIES OF CHILDREN WITH MUSCULAR DYSTROPHIES: BURDEN, SOCIAL NETWORK AND PROFESSIONAL SUPPORT

Politano Luisa (2), Stutifero Marianna (2), Zaccaro Antonella (2), Balottin Umberto (3), Berardinelli Angela Lucia (3), Camia Michela (3), Motta Maria Chiara (3), Vita Giuseppe (4), Messina Sonia (4), Sframeli Maria (4), Vita Gianluca (4), Pane Marika (5), Lombardo Maria Elena (5), Scalise Roberta (5), D'Amico Adele (6), Catteruccia Michela (6), Colia Giulia (6), Angelini Corrado (7), Gaiani Alessandra (7), Semplicini Claudio (7), Battini Roberta (8), Astrea Guja (8), Ricci Giulia (8), D'Angelo Maria Grazia (9), Brighina Erika (9), Civati Federica (9), Patalano Melania (1), Sagliocchi Alessandra (1), Magliano Lorenza (1)

(1) Dipartimento di Psicologia, Seconda Università di Napoli, Viale Ellittico, 31, 81100 Caserta, Tel. 0823.275358; Fax 0823.274759; E-mail:lorenza.magliano@unina2.it
(2) Cardiomiologia e Genetica Medica, Dipartimento di Medicina Sperimentale, Seconda Università di Napoli
(3) Unità di Neuropsichiatria infantile, IRCSS "C. Mondino", Pavia
(4) Dipartimento di Neuroscienze, Scienze Psichiatriche e Anestesiologiche, Università di Messina
(5) Dipartimento di Neuropsichiatria Infantile, Università Cattolica, Roma
(6) Unità per i Disordini Neuromuscolari e Neurodegenerativi, Dipartimento di Neuroscienze, Ospedale Bambin Gesù, Roma
(7) Dipartimento di Neuroscienze, Università di Padova
(8) Dipartimento di Neurologia, Università di Pisa
(9) Unità Neuromuscolare-Dipartimento di Neuroriabilitazione, IRCCS E. Medea, Bosisio Parini, Lecco

Muscular Dystrophies (MD) are chronic, degenerative diseases which may led to marked functional impairment and reduced life expectancy, highly affecting the quality of life of patients and their relatives. Patients' relatives, particularly in the late stages of MD, deal with very high level of practical and psychological burden of care. This burden, as well as social and professional factors which may mitigate it, are largely unexplored.
GUP10002 study aims to describe the burden experienced by the families of patients with MD in Italy, and the social and professional resources they may rely on. The study is carried out on key-relatives (18-80 year old) of patients suffering from Duchenne, Becker, or Limbe-girdle MD, aged between 4 and 25 years, followed for at least 6 months in one of 8 participating Centres, located all over Italy. Moreover, the study aims to explore the burden perceived by the 9-17 years-old healthy siblings closest in age to the patient. Data have been collected by using standardised assessment instruments.
The study, started in 2011, includes the following 3 phases:
1) Preparatory phase (6 months): in this phase the following goals have been achieved:
a) Finalization of the protocol and adaptation of questionnaires on family burden, social network and professional support to MD; development of an ad-hoc schedule on treatments received by the patients and on the support provided to their relatives by each Centre; development of a semi-structured interview to assess the patient's levels of functional autonomy according to Barthel Index (BI) scoring; development of a guide for the researchers.
b) Development of databases and of a guide for the data entry.
c) Training courses of researchers in the use of the assessment tools and data input. Nineteen researchers attended the 2 courses held in Naples on September and October 2011. In the training, inter-rater reliability in the use of the BI interview was formally measured.
d) Screening of eligible cases stratified for diagnosis. In the 8 Centres, a total of 1056 patients with MD were found, with a number of patients in charge per Centre ranging from 43 to 344. Among them, Duchenne patients were 32-92%; Becker patients were 8-58%; and LGMD were 0-32%. Accordingly to the type of the Centres – paediatrics, adults, or combined – the percentage of minor-aged relatives varied from 100 to 32%.
2) Main phase of data collection (12 months): Up to 30th September 2012, 408 key-relatives have been consecutively enrolled, 288 of them were relatives of DMD patients, 93 were relatives of BMD patients, and 27 relatives of LGMD patients. Furthermore, 17 young healthy siblings have been interviewed. Data collection will be completed in December 2012.
3) Analysis of data and dissemination of results (6 months). These phases will be carried out in 2013. Study results will be used to develop targeted supportive family interventions in MDs.

ABSTRACT N. 69

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BIANCHI MARIA LUISA**	
Telethon grant N.	**GUP11011**	
Total budget €	**380.900**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

EVALUATION OF BONE TURNOVER, BONE METABOLISM, BONE DENSITY, AND FRACTURES IN CHILDREN WITH DUCHENNE MUSCULAR DYSTROPHY AND POSSIBLE SIDE EFFECTS OF LONG-TERM CORTICOSTEROID THERAPY (BON-DMD)

Maria Luisa Bianchi

Centro Malattie Metaboliche Ossee, Istituto Auxologico Italiano IRCCS, via Ariosto 13, 20155 Milano, Tel. 02.619112505, Fax 02.619112519, E-mail: ml.bianchi@auxologico.it

The project (BON-DMD) is ancillary to an international multicenter

project (FOR-DMD), already independently financed, starting in December 2012-January 2013.
The study object is one of the most frequent X-linked genetic diseases, Duchenne Muscular Dystrophy (DMD). The main aim of FOR-DMD is the evaluation of three different therapeutic regimens based on corticosteroids (CS), the only treatment that, even if not resolutive, has demonstrated positive effects in DMD, regarding both preservation of muscular function and prolongation of life.
Unfortunately, long-term, high-dose use of CS has several negative side effects, often severe, including the early development of secondary osteoporosis (reduction of bone mineral mass and micro-architectural deterioration, with increased bone fragility and risk of fractures).
The study will be conducted in 40 Centers located in 5 different countries (Italy, Germany, UK, Canada, USA). 300 children (4-7 years) affected by DMD, all ambulant, prepubertal, not yet on CS treatment will be enrolled.All the enrolled patients will complete a 36-month follow-up (the enrollment period will be of 24 months). Bone metabolism and density, as well as fractures, will be evaluated at baseline and every year thereafter. Children will be randomly assigned to one of three different CS regimens (daily vs. intermittent prednisone and deflazacort).
Within the BON-DMD study, all the 300 patients enrolled will undergo a thorough evaluation of bone metabolism and turnover, through the analysis of specific biochemical "bone markers", as well as a more accurate study of the evolution of bone mass. Moreover, all incident fractures will be recorded. The unique opportunity of studying a large population of prepubertal DMD patients is expected first to answer the unresolved question about whether DMD per se, even before CS use and independently of it, causes loss of bone mass and weakening of bone structure, and second to provide clues to the identification of children at higher risk of bone loss and fractures.
For the first time, different parameters (including bone turnover markers and cytokines) will be used to identify the patients at high risk of low BMD, increased bone loss or fractures during CS treatment. Moreover, parathyroid hormone (PTH) and 25-OH vitamin D will be measured as the main regulators of calcium metabolism, as well as for their physiological interaction (feedback regulation of PTH secretion by vitamin D). In DMD, increased bone resorption because of vitamin D deficiency and secondary hyperparathyroidism has been observed.
An additional benefit of BON-DMD will be the implementation of a strict quality control procedure to ensure that all the DXA measurements performed throughout the 60-month project at different and geographically dispersed participating Centers, with different instruments, will maintain the high quality, long-term stability and comparability required by such a large investigation and investment.
The study will be an ideal condition for evidence-based conclusions on the bone effects of DMD per se, before CS use, and will also offer a sound basis to evaluate the impact of CS treatment in a large number of DMD patients (100 for each CS regimen) and to identify the CS regimen with less adverse effects on bone. It is expected that the BON-DMD conclusions will lead to the development of an optimal strategy to maximize bone density, control bone turnover and reduce fracture risk in DMD.

ABSTRACT N. 70

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**TUPLER ROSSELLA GINEVRA**	
Telethon grant N.	**GUP11009**	
Total budget €	**200.000**	
Centres: **15**	*Duration (yrs):* **1**	*Starting year:* **2012**

DEVELOPMENT OF THE ITALIAN NATIONAL REGISTRY FOR FSHD

Ricci Giulia (2), Scionti Isabella (1), Sera Francesco (3), Govi Monica (1), Mele Fabiano (1), D'Amico Roberto (4), Filosto Massimiliano (5), Vercelli Liliana (6), Ruggiero Lucia (7), Berardinelli Angela (8), Angelini Corrado (9), Antonini Giovanni (10), Bucci Elisabetta (10), Cao Michelangelo (9), Di Muzio Antonio (11), Iannaccone Elisabetta (12), Maggi Lorenzo (13), Moggio Maurizio (14), Morandi Lucia (13), Mongini Tiziana (6), Pastorello Ebe (15), Ricci Enzo (12), Rodolico Carmelo (16), Santoro Lucio (7), Siciliano Gabriele (2), Tomelleri Giuliano (17), Galluzzi Giuliana (18), Tupler Rossella (1)

(1) Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, via G. Campi 287, 41125 Modena, Italy, Tel. 0592055414, Fax 0592055426, E-mail: rossella.tupler@unimore.it
(2) Department of Neuroscience, Neurological Clinic, University of Pisa, Pisa, Italy
(3) ICH - Paediatric Epidemiology Unit, Department of Population Health Sciences, Faculty of Population Health Sciences, London UK
(4) University of Modena and Reggio Emilia, Unit of statistics, Department of Oncology and Hematology, Italy
(5) Clinical Neurology, University Hospital "Spedali Civili", Brescia, Italy
(6) Department of Neuroscience, Center for Neuromuscular Diseases, University of Turin, Turin, Italy
(7) Department of Neurological Sciences, University Federico II, Naples, Italy
(8) Unit of Child Neurology and Psychiatry, IRCCS "C.Mondino" Foundation, Pavia, Italy
(9) Department of Neurosciences, University of Padua, Padua, Italy
(10) Department of Neurology, S. Andrea Hospital, University of Rome "Sapienza", Rome, Italy
(11) Center for Neuromuscular Disease, University "G. d'Annunzio", Chieti, Italy
(12) Department of Neurosciences, Università Cattolica Policlinico A. Gemelli, Rome 00168, Italy
(13) IRCCS Foundation, C. Besta Neurological Institute, Milano, Italy
(14) Department of Neurology, IRCCS Fondazione Ospedale Maggiore Policlinico, University of Milan, Milan, Italy
(15) Department of Neurological and Psychiatric Sciences, University of Padua, Padua, Italy
(16) Department of Neurosciences, Psychiatry and Anaesthesiology, University of Messina, Messina, Italy
(17) Department of Neurological Sciences and Vision, University of Verona, Verona, Italy
(18) Molecular Genetics Laboratory of UILDM, Lazio Section, IRCCS Santa Lucia Foundation, Rome, Italy

Facioscapulohumeral muscular dystrophy (FSHD) is classified as an autosomal dominant myopathy associated with deletions of integral copies of tandem 3.3 kilobase repeats (D4Z4) combined with 4A(159/161/168) PAS haplotype at 4q35. It has been estimated an almost full penetrance of the disease by the age of 20. However, our studies reported a unexpected high percentage of asymptomatic or minimally affected subjects carrying a reduced D4Z4 allele (DRA) at 4q (J Med Genet 49:171-8, 2012). Recently we have reported that 3% of healthy subjects carried alleles with reduced number (4-8) of D4Z4 repeats on chromosome 4q (Am J Hum Genet 90:628-35, 2012).
Through the Italian National Registry, 530 subjects carrying DRA (367 relatives and 167 probans) from 176 unrelated FSHD families were clinically and molecularly evaluated. Size of DRA, gender, age, degree of kinship and 4q haplotype were evaluated to establish the role of these variable on severity of the clinical expression. The FSHD scale was used to define muscle impairment, which can be translated into a number (FSHD score).
Overall, 32.2% of relatives did not display any muscle functional impairment, with 47.1% of unaffected individuals among second-through third-degree relatives and 27.5% among first-degree family members. The estimated risk of developing FSHD for relatives carrying DRA with 1-3 repeats was 64.3% at age 20, 80.1% at age 40, and 96.2% at age 60. The risk of FSHD for relatives carrying DRA with 4-8 repeats was approximately 20% at age 20, 43% at age 40, 65% at age 60.
Comparison of the FSHD score given to females versus males with DRA of the same size, displays that male relatives received a mean score significantly higher than females relatives (mean FSHD score: 5.4 in males vs 4.0 in females, p0.003). No 4q haplotype was exclusively associated with the presence of disease.
Our work establishes that DRAs with 4-8 repeats have limited prognostic value. In this class of alleles female gender and degree of kinship are associated with a reduced risk of developing FSHD. No specific haplotype is predictive of disease expression.
Collectively our data suggest that the pathogenesis of FSHD is more complex than previously thought and that the current genetic molecular signature needs to be carefully re-considered as predictor of disease outcome, with crucial consequences for genetic counseling and prenatal diagnosis.

ABSTRACT N. 71

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FIORILLO CHIARA**	
Telethon grant N.	**GEP12019**	
Total budget €	**35.300**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

EXPLORING MITOCHONDRIAL DYSFUNCTION IN CALPAIN-3 RELATED MUSCULAR DYSTROPHY

Baldacci Jacopo (1), Cassandrini Denise (1), Nesti Claudia (1), Mes-

chini Chiara (1), Mora Marina (2), Ruggiero Lucia (3), Santoro Lucio (3), Politano Lucia (4), Siciliano Gabriele (5), Santorelli Filippo (1), Fiorillo Chiara (1)

(1) Fondazione Stella Maris IRCCS - Viale del Tirreno 331, 56018, Calambone, Pisa, Tel. 050886311, Fax 050886247, E-mail: c.fiorillo@inpe.unipi.it
(2) Istituto Neurologico C.Besta, Milano
(3) Clinica Neurologica, Università di Napoli Federico II, Napoli
(4) Genetica Medica, Seconda Università di Napoli, Napoli
(5) Clinica Neurologica, Università di Pisa, Pisa

Background/Rationale:
Mutations in the CAPN3 gene, coding for the muscle specific isoform calpain-3, cause LGMD2A, a form of progressive muscular dystrophy of intermediate severity affecting children and young adults. The exact role of calpain-3 is still controversial and so are the consequences of its mutations. In fact, calpain-3 has been implicated in such diverse processes as apoptosis, modification of transcription factors, cytoskeletal and sarcomere rearrangements, sarcolemma stability and calcium handling. A recent study evidenced mitochondrial abnormalities, energy defect and oxidative stress in myoblasts from Capn3 knock-out mice (C3KO).
Broad objectives and specific aims:
This study aims to investigate pathological effects of calpain-3 mutations in muscle tissues from LGMD2A patients. In particular a putative mitochondrial dysfunction will be evaluated in terms of abnormalities of mitochondrial morphology, oxidative phosphorylation (OXPHOS), and promotion of oxidative stress. Results are expected to increase knowledge of calpain-3 disease mechanism in human muscle cells and might provide therapeutic means for patients with LGMD2A.
Research design and methods:
To address the question whether the mitochondrial alterations detected in mice also apply to LGMD2A patients, we will analyze cultured myoblasts in patients harboring mutations in CAPN3. Evaluation of respiratory chain enzymes, ATP content, mitochondrial membrane potential, and ROS production will be performed. We also plan to screen the results from pharmacological modulation of the putative energetic defect such as modulation of ATP content with cyclosporine A. Muscle biopsies from the same set of patients will be also tested in vitro for respiratory chain defects with standard biochemical procedures.
Preliminary results:
From our database we identified 7 patients with confirmed mutations in CAPN3 and collected 2 novel muscle biopsies. In one biopsy we could detect an altered COX staining and a reduced activity of chain respiratory complexes. We also obtained 3 additional myoblast cell lines from the Telethon Biobank.
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ABSTRACT N. 72

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BRUNO CLAUDIO**	
Telethon grant N.	**GEP12053**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

EXTRACELLULAR ADENOSINE-TRIPHOSPHATE (E-ATP) AND PURINERGIC SIGNALLING IN THE PATHOGENESIS OF ALPHA-SARCOGLYCAN DEFICIENT MUSCULAR DYSTROPHY (LGMD2D)

Baldassari Simona (1), Gazzerro Elisabetta (1), Traggiai Elisabetta (2), Grassi Fabio (3), Bruno Claudio (1)

(1) U.O. Neurologia Pediatrica e Malattie Muscolari, IRCCS Istituto Giannina Gaslini, Largo G. Gaslini 5, 16147 Genova, Italy, Tel. +39.010.5636756; Fax +39.010.3538265, E-mail: claudiobruno@ospedale-gaslini.ge.it
(2) U.O. Pediatria II-IRCCS Istituto Giannina Gaslini-Genova, Italy
(3) Dipartimento di Biologia e Genetica per le Scienze Mediche, Univ. degli Studi di Milano-Milano, Italy

The project aims to analyze the role of the extracellular ATP (eATP)/purinergic pathway in the dystrophic process due to alpha-sarcoglycan (alpha-SG)-deficiency and to evaluate the therapeutic potential of eATP signalling modulation through a purinergic (P2) receptor antagonist. Although the Limb Girdle Muscular Dystrophy caused by alpha-SG deficiency (LGMD2D) is not categorized as an inflammatory myopathy, the inflammatory response is an important pathological component of this disease.
eATP is a "danger" associated molecule involved in the priming and development of adaptive and innate immune response. Indeed, eATP, when released from the cytosol of dying cells, contributes in the initial phase of the immune response, to an efficient activation of Antigen Presenting Cells and priming of T cells while, later, it expedites the amplification of the inflammasome reaction. During chronic inflammation eATP levels increase and the inhibition of this pathway improves the phenotype of animal models with distinct inflammatory diseases. However, notably, eATP plays also a crucial role in the regulation of calcium homeostasis in muscle cells.
The pathogenetic role of eATP could even be more relevant in LGMD2D, since, very intriguingly, alpha-SG displays an ATP-binding site in its extracellular domain and is characterized by an ecto-ATPase activity thus controlling eATP concentration at the surface of cells expressing P2 receptors, attenuating the magnitude and/or the duration of eATP-induced signals. The absence of alpha-SG, and therefore loss of ecto-ATPase activity on the muscle sarcolemma, could cause a persistent increase of eATP concentration, thus amplifying its effects. The consequent prolonged stimulation by eATP of P2 receptors may in turn lead to intracellular Ca2+ overload and finally to cell death.
In our project, we aim at characterizing the eATP/purinergic pathway in primary cultures of myoblasts isolated from human and murine alpha-SG-deficient skeletal muscles. Thus, we will analyse eATP synthesis release, cleavage and P2 receptors expression and function in myoblasts. Moreover, we plan to test the effectiveness of an 'epigenetic drug', oATP, a P2 receptor antagonist, in a mouse model of alpha-SG deficiency. Clinical, histopathological, immunological parameters and calcium homeostasis will be evaluated in alpha-Sg null mice systemically treated with this drug in acute and chronic trials.
Our study will contribute to clarify the pathogenetic mechanisms leading to muscle cells degeneration in LGMD2D and will concur to the identification of a novel pharmacological strategy aimed at ameliorating muscle inflammation and calcium homeostasis in this disease.

ABSTRACT N. 73

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SANDONA' DORIANNA**	
Telethon grant N.	**GEP12058**	
Total budget €	**49.400**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

PHARMACOLOGICAL RESCUE OF MISFOLDED PROTEINS: INNOVATIVE APPROACHES FOR THE CURE OF MUSCULAR DISEASES

Bianchini Elisa (1), Gomiero Chiara (1), Dorotea Tiziano (2), Valle Giorgia (1), Mascarello Francesco (2), Betto Romeo (1,3), Volpe Pompeo (1), Sacchetto Roberta (2), Sandonà Dorianna (1)

(1) Department of Biomedical Sciences University of Padua, Via G. Colombo 3, 35121 Padua, Tel. +39 049 8276028, Fax +39 049 8276040, E-mail: dorianna.sandona@unipd.it
(2) Department of Comparative Biomedicine and Food Science, University of Padua
(3) Institute of Neuroscience, Padua, National Research Council of Italy

The key pathogenetic event of an extremely heterogeneous group of genetic diseases, collectively called Unfolded Protein Diseases (UPDs), is the presence of gene mutations that cause either unfolding or misfolding of a coded protein. This usually leads to either toxic gain of function, because of mutated protein aggregation, or loss of function because of the premature disposal of the defective protein by the cell's quality control system (QCS).
Sarcoglycanopathies, in particular type 2D and 2E Limb Girdle Muscular Dystrophy (LGMD2D/2E), Brody's disease (BD) and its bovine counterpart pseudomyotonia (PMT) and recessive Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) are genetic diseases

of striated muscles showing characteristics of UPDs. Despite presenting largely different clinical phenotypes, these disorders share a common feature, i.e., normal levels of the mutated transcripts and almost undetectable levels of the coded products (alpha/beta-sarcoglycan, SERCA1a and calsequestrin2, respectively). Evidence from our laboratory demonstrates that mutants causing LGMD2D (Gastaldello et al Am. J. Path. 2008) and PMT (ongoing results) are substrate of the ER associated protein degradation (ERAD).
Unfortunately, at present, no effective therapies are available for these disorders. Aim of this project is to prove the feasibility of a new pharmacological therapy designed "to cure" the mutated proteins of such pathologies either by preventing their degradation (protein rescue strategy) or promoting their folding and trafficking (protein repair strategy).
We have identified several ERAD components responsible for the degradation of the V247M alpha-sarcoglycan mutant that might be potential drug targets in the so called "protein rescue strategy". Knock down of selected ERAD components by using small interfering RNAs, in fact, permits the rescue of the alpha-sarcoglycan mutant. On the other hand, inhibition of proteasome has positive effects also on the recovery of SERCA1a mutant in both in vivo experiments on cellular model and ex vivo experiments on skeletal muscle explants isolated from a PMT affected cattle.
Regarding the "protein repair strategy", we are testing several different small molecules that, by assisting folding, promote the mutant proteins' maturation and trafficking. First experiments are very promising because drug treatments preserve both SERCA1a and alpha-sarcoglycan mutants from degradation and permit their proper localization.
The present project constitutes the proof of principle for the development of innovative pharmacological therapies for the cure of rare muscle diseases for which neither classical nor innovative therapies, i.e., gene therapy, are efficacious or applicable.

ABSTRACT N. 74

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BANG MARIE-LOUISE**	
Telethon grant N.	**GGP12282**	
Total budget €	**430.600**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2012**

MYOPALLADIN IN DILATED CARDIOMYOPATHY AND LIMB GIRDLE MUSCULAR DYSTROPHY

Ludwig Yamamoto Daniel (2,3), Marmonti Enrica (1), Caremani Marco (4), Chen Ju (5), Nigro Vincenzo (6,7), Linari Marco (4), Bang Marie-Louise (1)

(1) Institute of Genetic and Biomedical Research, National Research Council (IRGB-CNR), Milan Unit at Humanitas Clinical and Research Center, Via Manzoni 113, 20089 Rozzano, Milan. Tel. 02.82245210, Fax 02.82245290, E-mail: marie-louise.bang@cnr.it
(2) Institute of Biomedical Technologies, National Research Council (ITB-CNR)
(3) MultiMedica Hospital, Scientific and Tecnology Pole, Milan
(4) Department of Biological Evolution, University of Florence, Florence
(5) Department of Medicine, University of California San Diego, USA
(6) Telethon Institute of Genetics and Medicine (TIGEM), Naples
(7) Department of General Pathology, Second University of Naples

Myopalladin (MYPN) is a striated muscle-specific sarcomeric protein, which belongs to the palladin/myopalladin/myotilin family of actin-associated immunoglobulin-containing proteins. Within the Z-line of skeletal muscle, MYPN binds to alpha-actinin, nebulin, and PDZ-LIM proteins (Bang et al. J Cell Biol 153, 413-27, 2001; von Nandelstadh et al. Mol Cell Biol 29, 822-34, 2009). Furthermore, MYPN in present in the nucleus and the I-band, where it binds to the stress-inducible transcriptional cofactor Cardiac Ankyrin Repeat Protein (CARP), which, in turn, binds to the I-band region of titin, suggesting a role of MYPN in mechanosensing (Miller et al. J Mol Biol 333, 951-64, 2003). The important role of MYPN in striated muscle is illustrated by the recent identification of MYPN mutations in human dilated, hypertrophic, and restrictive cardiomyopathy (Duboscq-Bidot et al. Cardiovasc Res 77, 118-25, 2008; Purevjav et al. Hum Mol Genet 21, 2039-53, 2012; Meyer et al. Eur J Hum Genet. Epub 2012). In addition, we recently identified MYPN mutations in limb girdle muscular dystrophy patients with associated dilated cardiomyopathy.
The aim of the project is to use adeno-associated virus (AAV) to deliver wildtype (WT) and mutant MYPN to MYPN knockout (MKO) in vivo, allowing us to test whether the identified mutations are causative for disease and whether delivery of WT MYPN can rescue the phenotype of the MKO mice.
In our studies of MKO mice we have found that MKO mice are significantly smaller compared to their wildtype (WT) littermates with an up till 40% reduction in skeletal muscle fiber cross-sectional area. Consistently, reduced differentiation rate and myotube width was observed in primary skeletal muscle cultures derived from MKO mice and MKO muscle exhibited reduced isometric force and power in proportion to the reduced fiber size. By up- and downhill treadmill running, MKO and WT mice performed similarly. However, while the performance of WT mice was unchanged following four consecutive days of downhill running (eccentric contractions), the performance of MKO mice decreased progressively and signs of muscle regeneration following muscle damage were observed. Consistent with a higher susceptibility to muscle damage, progressive Z-line widening was observed in MKO skeletal muscle from about eight months of age. Finally we found that MYPN can directly bind to and bundle filamentous actin and plays a role in activation of the serum response factor (SRF) pathway, which is regulated by changes in actin dynamics and has been shown to be required for skeletal muscle growth and maturation (Li et al. PNAS 102, 1082-1087, 2005). MYPN's role in organizing the actin cytoskeleton and in SRF-induced signaling may provide an explanation for the smaller skeletal muscle fiber size, reduced differentiation rate, and decreased muscle performance in MKO mice.

ABSTRACT N. 75

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FIMIA GIAN MARIA**	
Telethon grant N.	**GEP12072**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

IS THE LIMB-GIRDLE MUSCULAR DYSTROPHY TYPE 2H A DEFECTIVE AUTOPHAGY DISEASE?

Romagnoli Alessandra (1), Di Rienzo Martina (1), Fusco Carmela (2), Micale Lucia (2), Merla Giuseppe (2), Fimia Gian Maria (1)

(1) National Institute for Infectious Diseases 'L. Spallanzani' IRCCS, Via Portuense 292, 00149 Rome, Italy. Tel. +390655170908, Fax +39065582825, E-mail: gianmaria.fimia@inmi.it
(2) Medical Genetics Unit, IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo, Italy

Autophagy is an intracellular catabolic process which plays an essential role in tissue homeostasis by removing damaged proteins and organelles. Optimal autophagic activity has been shown to be crucial for the maintenance of muscle integrity, with low levels of autophagy leading to muscle degeneration and high levels to loss of muscle mass.
LGMD2H is a mild, autosomal recessive muscle disease clinically characterized by a slowly progressive proximal weakness and wasting. LGMD2H is caused by mutations of the trim32 gene, however the molecular mechanisms responsible for the onset of the disease remains largely unknown.
The aim of this study is to elucidate whether defective autophagy contributes to the onset of the limb-girdle muscular dystrophy type 2H (LGMD2H). By a functional proteomic approach, we have recently found that TRIM32 interacts with the autophagy regulator Ambra1. Prompted by this result, we are currently testing whether the ubiquitin E3 ligase activity of TRIM32 regulates Ambra1 function and plays a role in the protective function of autophagy in muscle cells. Moreover we are testing whether mutations of the trim32 gene found in LGMD2H patients interfere with the binding to Ambra1 and cause a less efficient turnover of muscle components mediated by autophagy.
Altogether, these approaches will contribute to understand whether muscle degeneration observed in LGMD2H patients is associated with defective autophagy.

ABSTRACT N. 76

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PETRUZZELLA VITTORIA**	
Telethon grant N.	**GEP12025**	
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Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

IDENTIFICATION OF THE GENE DETERMINING LIMB GIRDLE MUSCULAR DYSTROPHY TYPE 1H

Bianco Angelica (1), Bisceglia Luigi (2), Amati Angela (3), Zoccolella Stefano (3), Petruzzella Vittoria (1)

(1) Dipartimento di Scienze Mediche di Base, Neuroscienze ed Organi di Senso, Università degli Studi "Aldo Moro", Piazza G. Cesare, 70124, Bari, Tel 080.5448529, Fax 080.5448538, E-mail: vittoria.petruzzella@uniba.it
(2) Ospedale Casa Sollievo della Sofferenza IRCCS, UOC Genetica Medica, San Giovanni Rotondo, Italia
(3) Azienda Ospedaliera-Universitaria Policlinico Consorziale, Raggruppamento di Scienze Neurologiche, Bari

We propose to identify the genetic features of a large pedigree affected by an autosomal dominant form of Limb-Girdle muscular dystrophy (LGMD), LGMD1H (Bisceglia et al., 2010 A new locus on 3p23-p25 for an autosomal-dominant limb-girdle muscular dystrophy, LGMD1H. EurJHumGenet. 6:636-641). We had previously mapped the LGMD1H locus on chromosome 3p23-25 in a four-generation Italian family presenting a slow progression of proximal muscle weakness in both upper and lower limbs and a relatively benign course with onset during the fifth decade of life.
Having excluded several candidate genes in the 3p23-25 region by conventional Sanger sequencing, the aim of the present proposal is to identify the disease gene by whole-exome HT-NGS (GA-IIe platform, Illumina). Blood DNA samples from five family members (generation 2: 1 healthy, 2 affected individuals; generation 3: 1 healthy and 1 affected) were sheared by Bioruptor and libraries were prepared by SPRIWorks FX-library method on robot Beckman FX using TruSeq Illumina adapters. Exome capture was performed with the TruSeq Exome Enrichment Kit (Illumina) which targets about 62 Mbp of human genome. Pair-end sequencing was performed on the HiSeq2000 (Illumina) generating 100-base pair-end reads. The following are the raw million reads (mr) produced for each patient: L1 = 61.9 million reads; L2 = 60.5 mr; L3 = 52.8 mr; L4 =59.4 mr; L5 = 65.1 mr. Sequence alignment, variant calling, and annotation are currently ongoing. Paired-end reads are now in the exome analysis pipeline at IGA Technology Services, consisting of base calling using Illumina Pipeline, mapping and alignment on the human genome reference sequence (NCBI build 37/UCSC hg19), selecting uniquely mapping reads with proprietary script, PCR duplication removal for single nucleotide variants and indel calling. To distil the causative mutation from the plethora of expected changes in exome sequencing, variants will be predicted in silico for their damaging role, segregation in the family will be tested, and mutations will be ruled out in ethnically-matched controls.
By the combination of new sequencing technologies with more traditional genetic techniques, a limited number of changes with disease relevance should emerge to give a genetic diagnosis of LGMD1H. This result will be a step forwards in the attempt to characterize all the key genes involved in the process of muscle degeneration in the several forms of LGMD.

ABSTRACT N. 77

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**NIGRO VINCENZO**	
Telethon grant N.	**GUP11006**	
Total budget €	**240.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

GENETIC DIAGNOSIS OF ITALIAN LGMD PATIENTS BY NGS TECHNOLOGY

Nigro Vincenzo (1,2), Savarese Marco (1,2), Mutarelli Margherita (2), Torella Annalaura (1), Di Fruscio Giuseppina (1), Politano Luisa (3), Fanin Marina (4), Pegoraro Elena (4), Angelini Corrado (4), Bruno Claudio (5), Minetti Carlo (5), Santoro Lucio (6), Comi Giacomo (7), Toscano Antonio (8), Siciliano Gabriele (9), Bertini Enrico (10), D'Amico Adele (10), Mongini Tiziana (11), Ricci Enzo (12), Belardinelli Angela (13), Santorelli Filippo Maria (14), Fiorillo Chiara (14), Sacconi Sabrina (15), Del Vecchio Blanco Francesca (1), De Concilio Ombretta (1), Piluso Giulio (1), Dionisi Manuela (2), Giugliano Teresa (1), Iacomino Michele (1), Garofalo Arca (1), Erpice Rosalba (1), Cuomo Anna (1), Pisano Cristina (1), Cutillo Luisa (2), Guarracino Mario (16)

(1) Dipartimento di Biochimica, Biofisica e Patologia Generale, Seconda Università degli Studi di Napoli - Laboratorio di Genetica Medica, via Luigi De Crecchio 7, 80138 Napoli, Italy, Tel. 0815665704, E-mail: vincenzo.nigro@unina2.it
(2) Telethon Institute of Genetics and Medicine, via Pietro Castellino 111, Napoli, Italy
(3) Cardiomiologia e Genetica Medica, Seconda Università degli Studi di Napoli, Napoli, Italy
(4) Dipartimento di Neuroscienze, Università degli Studi di Padova, Padova and IRCSS S. Camillo, Venezia, Italy
(5) Università di Genova e Unità malattie muscolari, Opedale G. Gaslini, Genova, Italy
(6) Dipartimento di Scienze Neurologiche, Università di Napoli "Federico II", Napoli, Italy
(7) Dipartimento di Scienze Neurologiche, Università degli Studi di Milano, Italy
(8) Dipartimento di Neuroscienze, Università di Messina, Messina, Italy
(9) Dipartimento di Neuroscienze, Università di Pisa, Pisa, Italy
(10) Ospedale Ped. Bambino Gesù, Roma, Italy
(11) AOU S. Giovanni Battista, SS Malattie Neuromuscolari, Torino, Italy
(12) Dip di Neurologia, Policlinico Gemelli, Università Cattolica, Roma, Italy
(13) IRCCS Mondino, Pavia, Italy
(14) IRCCS Stella Maris, Pisa, Italy
(15) Nice Hospital and UMR CNRS6543, Nice University, Nice, France
(16) National Research Council of Italy (ICAR-CNR), Napoli, Italy

Next generation sequencing (NGS) has a tremendous impact on our knowledge of different aspects of biology. It can be also very powerful to study patients with heterogeneous genetic conditions, like limb-girdle muscular dystrophies (LGMD).
Our project aims to diagnose mutations in all the known causative genes by targeted NGS approach and CGH array, and to discover new genes using "exome resequencing" and RNASeq approaches. In the course of this first year of the project, we have recruited 150 DNA samples from Italian LGMD patients. LGMD patients to be studied in the present application have been selected according to the following criteria: a) clinical diagnosis of LGMD with proximal weakness and autosomal inheritance (both recessive and dominant); b) inconclusive molecular diagnoses after the complete gene testing of the known LGMD genes; c) severity of disease; d) availability of a muscle biopsy for mRNA extraction.
All DNA samples were first enriched for 486,480 bp covering 2,447 exons of 98 genes by using the Haloplex technology with the use of barcodes (Motor Haloplex). We then performed pooled NGS using the Illumina HiSeq1000 platform, by analyzing 128 samples grouped in 16 combinatorial pools at 2x75bp. We sequenced all 16 pools at ~ 3,000 x coverage and identified about 70 unique mutations, also confirmed by Sanger sequencing . Negative cases were then studied by the Agilent MotorChip CGH array version 3.0 to identify possible deletions or duplications. Our results confirm that there is a high genetic heterogeneity in limb-girdle muscular dystrophies and that NGS-based DNA testing are ready for diagnostic use.
We next used NGS to discover new genes by exome sequencing in families. Analysis consisted of at least 55Mb of sequences covering all recognized human transcripts. As an example, we investigated the seven-generation family with 64 members affected that was previously defined as LGMD1F. We sequenced the whole exome of four family members separated by up to eleven meioses and identified a single shared novel heterozygous frame-shift variant. This causes a nonstop change in the Transportin 3 (TNPO3) gene that encodes a member of the importin-ß super-family.
In conclusion, using different NGS approaches we expect to provide molecular diagnosis in up to 80% of all cases.

ABSTRACT N. 78

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**COMI GIACOMO PIETRO**	
Telethon grant N.	**GUP10006**	
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Centres: **8**	*Duration (yrs):* **2**	*Starting year:* **2011**

CLINICAL AND LABORATORY NETWORK FOR LGMD DIAGNOSIS, IN VIEW OF A NATIONAL REGISTRY

Magri Francesca (1), Nigro Vincenzo (2,3), Angelini Corrado (4), Mongini Tiziana (5), Mora Marina (6), Moroni Isabella (6), Toscano Antonio (7), Tomelleri Giuliano (8), Siciliano Gabriele (9), Comi Giacomo (1)

(1) Dipartimento di Scienze Neurologiche, Università degli Studi di Milano, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, via F. Sforza 35, 20122 Milano. Tel +39.02.55033817, Fax +39.02.55033800; E-mail: giacomo.comi@unimi.it
(2) Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, Napoli
(3) Telethon Institute of Genetics and Medicine (TIGEM), Napoli
(4) Dipartimento di Neuroscienze: SNPSRR, Università di Padova, Padova
(5) Dipartimento di Neuroscienze, AOU S. Giovanni Battista di Torino, Torino
(6) Divisione di Malattie Neuromuscolari e Neuroimmunologia, Fondazione IR-

CCS Istituto Neurologico C. Besta, Milano
(7) Dipartimento di Neuroscienze, Università di Messina
(8) Dipartimento di Scienze Neurologiche, Verona
(9) Dipartimento di Neuroscienze, Università di Pisa, Pisa

Limb girdle muscular dystrophies (LGMD) are heterogeneous disorders characterized by predominant limb girdle weakness with infantile and adult onset. They present high genetic variability: in recent years several new genes have been described leading to the definition of 18 autosomal recessive (AR) and 8 autosomal dominant (AD) forms, but about 30-40% of LGMDs still remains without molecular diagnosis. However advances in molecular genetic have stressed the importance of clinical-genetic correlations both for genetic counselling and for insight into pathogenesis.
The first aim of this project is to evaluate the clinical and genetic features of a large group of Italian LGMD patients in order to create an Italian network, define LGMDs epidemiology and delineate the basis of the creation of a national registry. In the second part an observational study over a 2 years-period will allow to define clinical history among different forms and to point out the most important clinical parameters which could be used in follow-up and to evaluate the efficacy of therapies.
As this condition is relatively rare, patients were enrolled from 8 different neuromuscular centres in order to obtain a larger sample. We collected 281 patients, 42 presenting with AD and 192 affected with AR forms. The most frequent AR forms were LGMD2A (17%) and LGMD2B (16%), followed by LGMD2I (11%), LGMD2D (9%), LGMD2E (4%), LGMD2C (3%), LGMD2L (2%) and LGMD2F (0.5%). Interestingly in one LGMD subject showing white matter abnormalities at brain MRI we found a LAMA2 gene mutation. In 49 patients mutations in known genes were not found.
Each form has a different severity and natural history. Mean age of onset is 27 years, LGMD2D having the most precocious onset (11.5±7.8 years). Creatine-kinase values are always markedly increased, especially in LGMD2B and 1C while LGMD1B showed the lowest values (295±127 UI/L).
Cardiac involvement, characterized by cardiomyopathy, is more frequently seen in LGMD2E and LGMD2I patients (respectively 30% and 44%), while in LGMD2A and 2B it is uncommon and is mainly characterized by arrythmias (6 and 21%). A mild respiratory restrictive pattern is generally present in 20% of cases, with exception of LGMD2E and LGMD2I which show a more severe involvement and a higher frequency (70 and 50%). Overall about 30% of patients are wheelchair bound by a mean age of 25 years. Muscle involvement, expressed as age of independent ambulation loss, is more severe in LGMD2D and 2A.
A two years observational study is currently ongoing in order to better establish the natural history and the parameters which can be used in its definition.
Overall this study will provide the fully characterization of this group of muscle disorders, improve our diagnostic capability, establish the spectrum of clinical/morphological/muscle imaging findings, explore genetic modifiers of clinical variability; these results might be useful to establish a baseline for potential therapeutic approaches.
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ABSTRACT N. 79

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MERCURI EUGENIO**	
Telethon grant N.	**GUP11001**	
Total budget €	**203.300**	
Centres: **13**	*Duration (yrs):* **2**	*Starting year:* **2012**

DEVELOPMENT OF A REGISTRY AND A DATABASE FOR A NATION-WIDE ITALIAN COLLABORATIVE NETWORK ON CONGENITAL MUSCULAR DYSTROPHY

Mercuri Eugenio (1), D'Amico Adele (2), Messina Sonia (3), Politano Luisa (4), Santorelli Filippo M. (5), Bruno Claudio (6), Boffi Patrizia (7), Berardinelli Angela Lucia (8), Pegoraro Elena (9), Moroni Isabella (10), Pini Antonella (11), Comi Giacomo Pietro (12), Corbo Massimo (13), Bianco Flaviana (1), Pane Marika (1)

(1) Child Neurology and PsichiatryUnit Institute of Psychiatry Catholic University Policlinico Gemelli Roma, Tel. 06 30155586, E-mail: eumercuri@gmail.com
(2) Neuromuscular and Neurodegenerative Unit, Department of Neuroscience, Bambino Gesù Children's Hospital, Rome
(3) Dipartimento di Neuroscienze, Scienze Psichiatriche e Anestesiologiche, Messina
(4) Department of Experimental Medicine, Second University of Naples
(5) Dipartimento di Medicina Molecolare, Neurogenetica e Malattie Neuromuscolari IRCCS Fondazione Stella Maris, Calambrone, Pisa
(6) Dept. of Pediatric Sciences, Istituto Giannina Gaslini, University of Genova
(7) Dipartimento di Scienze Pediatriche e dell'Adolescenza Sezione di Neuropsichiatria Infantile, Torino
(8) Fondazione "Istituto Neurologico Nazionale C. Mondino"Struttura Complessa di Neuropsichiatria Infantile Pavia
(9) Department of Neurosciences, University of Padova
(10) Division of Child Neurology, Fondazione IRCCS Istituto Neurologico C. Besta, Milano
(11) Unità Operativa di Neuropsichiatria Infantile, Dipartimento di neuroscienze, Azienda USL di Bologna. Bologna
(12) Department of Neurological Sciences University of Milano Milano
(13) NEMO (NEuroMuscular Omnicentre) Fondazione Serena Onlus, Ospedale Niguarda Ca' Granda, Milano

In the last 15 years the identification of several new genes responsible for different forms of congenital muscular dystrophies (CMD) has not only expanded the spectrum of the known forms of CMD but has produced exciting progresses in the understanding of the mechanisms underlying this group of disorders. Our ability to treat these disorders however is still extremely limited. Moreover, considering that CMDs are rare diseases, small patient number represents the major impediment to progress in research and care. This limitation can effectively be overcomed by harmonizing a nation-wide network to an international network for a patient register in combination with an effort to share assessments and outcome measures in order to aquire reliable natural history data. The principal goal is to converge toward a web-based register of patients with CMDs to better understand the phenotypes and to increase information on the natural history of these diseases.
In particular, this goal should be reached by
1) establishing an Italian network of all clinical centers with expertise on CMDs, and creating a registry of CMD cases converging toward the international web-based database made available by CURE CMD Association (CMDIR)
2) collecting epidemiological information on the distinct forms of CMD
3) using the registry as a platform for promoting standards of care and, in synergy with TREAT-NMD and CURE CMD, identifing the most suitable outcome measures for each form of CMD and use them in order to collect natural history data.
We believe that these fundamental steps are necessary to better understand the natural history of CMDs and, finally, to improve the management of these disorders and plan appropriate preliminary steps in preparation for possible therapeutic strategies.
In the first part of the study preliminary meetings have helped to identify patients actross the participating centers and to identify the best strategies to enrol all the CMD patients.
Considering the difficulties of the entries of the registry and that several Italian families have little experience with computers, alternative methods have ben discussed. It has been suggested that a pronted copy of the survey could be sent before consulation to the families so that they can start filling the paper version and discuss at the time of consultation the fields which were found to be difficult.
A translation of the full registry has just been completed and will be made available electronically in the next few months.

ABSTRACT N. 80

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**GAZZERRO ELISABETTA**	
Telethon grant N.	**GEP12046**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

DRUG DISCOVERY FOR DYSTROGLYCANOPATHIES VIA LARGE PROMOTER ACTIVATION SCREENING

Assereto Stefania (1), Galietta Luis (2), Zara Federico (1), Bruno

Claudio (1), Minetti Carlo (1), Gazzerro Elisabetta (1)

(1) Center of Myology, Pediatric Neurology Unit, Department of Neuroscience and Rehabilitation, G. Gaslini Institute, Largo G. Gaslini 5, 16147 Genova, Italy, Tel. +39.010.5636606; Fax +39.010.3538265; E-mail: eligazzerro@gmail.com
(2) Laboratory of Molecular Genetics, Istituto Giannina Gaslini, Genova

The project aims to develop and validate a cell-based high-throughput screening (HTS) assay for promoter activation of the glycosyltransferase LARGE gene. The term "dystroglycanopathies" is used to describe a number of heterogeneous Congenital Muscular Dystrophies (CMDs), which frequently include structural central nervous system (CNS) abnormalities and which encompass a striking range of clinical severity. Dystroglycanopathies are caused by alpha-dystroglycan (a-DG) hypoglycosylation. This transmembrane protein, once glycosylated in the mucin domain, binds extracellular ligands (laminin, agrin, perlecan) playing a critical role in the maintenance of basement membranes in muscle and non muscle tissues.
Defects in a total of 8 glycosyltransferases or accessory proteins cause a dystroglycanopathy phenotype (LARGE, POMT1, POMT2, POMGnT1, FKTN, FKRP, DMP2, DMP3).
The disease-severity of muscular dystrophy and the relative ratio of glycosylated versus hypoglycosylated a-DG molecule closely correlate. Patients at the severe end of the clinical spectrum tend to show the greatest reduction in a-DG glycosylation, while even a small amount of glycosylated protein is sufficient to preserve skeletal muscle function. Notably, different authors have shown that overexpression of LARGE can functionally bypass the glycosylation defect caused by mutations of other glycosyltransferases and can increase a-DG laminin affinity with a reduction of muscle disorder.
Thus, the identification of compounds able to up-regulate LARGE expression represents an attractive pharmacological strategy for dystroglycanopathies. The HTS of library of chemical compounds is an alternative approach to gene therapies aimed to up-regulate a target gene. The use of small, drug-like molecules to achieve LARGE upregulation offers obvious advantages in terms of delivery, bioavailability and immunological reactions.
The objective of our exploratory grant is the development of a robust, reproducible and validated cell-based assay for the activation of LARGE promoter. This is a critical step for the success of the screening of compounds or existing drugs which will be pursued in subsequent experiments. To develop the project, we will analyze LARGE promoter regions by a bioinformatic approach and then test them through stepwise deletion constructs of LARGE 5'sequences fused with a luciferase reporter in C2C12 cells (aim #1). Contemporarily, we will test positive controls, i.e. molecules able to increase LARGE transcripts (#2). Hence, we will generate and validate stable C2C12 cell lines expressing LARGE promoter regions/luciferase reporter (#3).

ABSTRACT N. 81

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PREVITALI STEFANO CARLO**	
Telethon grant N.	**GGP12024**	
Total budget €	**290.900**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

ROLE OF JAB1 IN THE CONTROL OF NERVE DEVELOPMENT AND REPAIR: IMPLICATION IN THE PATHOGENESIS OF MEROSIN DEFICIENT CONGENITAL MUSCULAR DYSTROPHY (MDC1A)-ASSOCIATED HEREDITARY NEUROPATHIES

Porrello Emanuela (1), Rivellini Cristina (1), Dina Giorgia (1), Velardo Daniele (1), Domi Teuta (1), Del Carro Ubaldo (1), Ungaro Daniela (1), Panattoni Martina (2), Feltri Maria Laura (3), Wrabetz Lawrence (3), Pardi Ruggero (2), Quattrini Angelo (1), Previtali Stefano Carlo (1)

(1) Division of Neuroscience and Institute of Experimental Neurology (INSPE), San Raffaele Scientific Institute, Via Olgettina 60, 20132 Milano, Tel 02.26433036, E-mail: previtali.stefano@hsr.it
(2) Division of Immunology, San Raffaele Scientific Institute, Milano, Italy
(3) Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Mlano, Italy

Merosin Deficient Congenital Muscular Dystrophy 1A (MDC1A) is a multiorgan disorder characterized by muscular dystrophy and dysmyelinating neuropathy. It is due to mutation in the LAMA2 gene, which encodes for laminin2, the main component of the neuromuscular basal lamina. Loss of laminin2 leaves orphan laminin2-receptors at the cellular surface, thus promoting tissue degeneration. In the previous Telethon grant application (GGP08037) we evaluated possible treatment of MDC1A by mesoangioblast stem cell transplantation, which were engineered to synthesize and secrete mini-agrin molecule (mMAG). mMAG was able to crosslink laminin2 orphan receptors at the muscle surface with the basal lamina and to partially rescue the disease in MDC1A mouse models. In fact, we observed amelioration of the muscular dystrophy, whereas peripheral neuropathy progressively worsened as well as motor performances of these mice.
In the present grant proposal, we are trying to better characterize the molecular machinery responsible for the generation of neuropathy associated-MDC1A, and in general of CMT neuropathies related to laminin2 signals. Defective Axonal sorting and dysmyelination are hallmark of MDC1A-associated neuropathies. Pathogenetic mechanisms include impaired Schwann cell proliferation, survival and differentiation and/or control of actin cytoskeleton. Jab1 is the catalytic component of the COP9 signalosome complex modulated by extracellular signals and involved in the control of cell cycle, protein degradation and gene expression. Jab1 is expressed in Schwann cells and modulated during nerve development and injury. By Cre/LoxP technology, we generated mice with conditional inactivation of Jab1 in Schwann cells. Jab1-null mice developed a peripheral neuropathy with histological characteristics similar to those observed in MCD1A-neuropathies. Moreover, Jab1 expression is reduced in mouse models of MDC1A-neuropathies. All these findings suggest that Jab1 acts downstream the laminin2 pathway and may be involved in the pathogenesis of MDC1A-neuropathies. During this grant application the role of Jab1 will be characterized in conditional knockout mice and in vitro cultures. We will investigate the molecular mechanisms responsible for defective axonal sorting, dysmyelination and nerve regeneration. Genetic rescue experiments to confirm pathogenetic mechanisms and to potentially treat MDC1A neuropathies will be also performed.
We expect to confirm that Jab1 is involved in the pathogenesis of MDC1A neuropathy and nerve regeneration. We envisage that exogenous modulation of Jab1 expression may ameliorate neuropathy in MDC1A mutants.

ABSTRACT N. 82

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FIUMARA FERDINANDO**	
Telethon grant N.	**GEP12087**	
Total budget €	**44.100**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

STRUCTURE-GUIDED THERAPEUTIC APPROACHES FOR OCULOPHARYNGEAL MUSCULAR DYSTROPHY (OPMD) AND RELATED DISEASES

Cibelli Marica, Pelassa Ilaria, Fiumara Ferdinando

Department of Neuroscience, Università degli Studi di Torino, Corso Raffaello 30, 10125 Torino, Tel. 011.6708486, E-mail: ferdinando.fiumara@unito.it

Oculopharyngeal muscular dystrophy (OPMD) is a human molecular disease caused by the expansion of DNA triplet repeats encoding for alanine in the PABPN1 gene. This expansion leads to the synthesis of PABPN1 proteins bearing longer homopolymeric alanine (polyA) stretches. OPMD belongs to a broader group of related genetic diseases similarly caused by polyA expansion in different genes. PolyA expansions induce pathological protein aggregation and dysfunction, leading to cell damage or death. OPMD manifests as a late-onset form of muscular dystrophy affecting primarily pharyngeal and ocular muscles with ptosis, dysphagia, dysphonia, eventually leading to limb weakness with more generalized motor impairment.
The mechanisms underlying the aggregation and toxicity of mutant PABPN1 are only partially understood, and are generally thought to be based on a structural misfolding of the protein induced by the polyA expansion, leading ultimately to the formation of aggregation-prone beta-sheet structures, in analogy with what observed in amyloid diseases. However, recent work in our laboratory has revealed a critical role for alpha-helical coiled coil (CC) structures in the aggregation and toxicity of polyA-expanded proteins. These observations prompted us to search for effective polyA CC-interference approaches that may result in novel therapies that are specific for OPMD and related diseases.
To this aim, we have developed a set of synthetic water-soluble peptides bearing polyA stretches of variable length, and we have

characterized their in vitro structure, stability, and oligomeric state through a combination of circular dichroism and chemical cross-linking techniques. The same peptides can also be overexpressed in cellular models to investigate their in vivo aggregation behavior. We are currently employing these peptides as a tool for systematic screenings in search of small molecule and peptide compounds capable of specifically inhibiting polyA aggregation in vitro and in vivo. Many of these compounds are produced through rational design based on their predicted structural features, and their capability to interact with specific structures in PABPN1 and other polyA-expansion proteins.
This work provides an effective platform for the screening and identification of compounds with therapeutic potential, specifically targeting the structural mechanisms of OPMD and other polyA-expansion diseases.

ABSTRACT N. 83

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CUBELLIS MARIA VITTORIA**	
Telethon grant N.	**GGP12108**	
Total budget €	**191.700**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2012**

PHARMACOLOGICAL CHAPERONES TO CURE GENETIC DISEASES: DEVELOPMENT OF DRUGS AND IDENTIFICATION OF NEW TARGETS

Andreotti Giuseppina (3), Cammisa Marco (1,2), Correra Antonella (1,2), Citro Valentina (1,2), Cimmaruta Chiara (3), De Crescenzo Agostina (4), Cubellis Maria Vittoria (1,2)

(1) Department of Structural and Functional Biology, University "Federico II", via Cinthia, Naples, Tel 3383484213, Fax 081679233, E-mail: cubellis@unina.it
(2) Istituto di Chimica Biomolecolare, CNR, Pozzuoli, Napoli
(3) Istituto di Biostrutture e Bioimmagini-CNR, Napoli
(4) Dipartimento di Scienze Ambientali, Seconda Università di Napoli, Caserta

The reduction in protein stability is the most common biochemical phenotype of monogenic diseases. This knowledge paves the way to a new therapeutic approach, pharmacological chaperones. These drugs are small molecules that stabilize the mutant proteins, increase their intracellular concentration and consequently their biologic activity. There is a great amount of data available for Fabry disease, a lysosomal storage disorder, which can be used as a valid model for experimental schedules. More than 400 mutations causing this disease have been described and we proved that responsive mutations, approximately 40% of the total can be predicted (Andreotti G, Guarracino MR, Cammisa M, Correra A, Cubellis MV: Prediction of the responsiveness to pharmacological chaperones: lysosomal human alpha-galactosidase, a case of study. Orphanet journal of rare diseases 2010, 5:36). Although this method is accurate, before proposing the pharmacological therapy in the place of Enzymatic Replacement therapy to patients affected by specific mutations, the prediction must be proved in vitro (Andreotti G, Citro V, De Crescenzo A, Orlando P, Cammisa M, Correra A, Cubellis MV: Therapy of Fabry disease with pharmacological chaperones: from in silico predictions to in vitro tests. Orphanet journal of rare diseases 2011, 6(1):66). The best thing would be to adapt a classic method to test protein thermodynamic stability on the mutants produced in the cells in a small quantity and unpurified. We propose to use the fusion induced by urea associated to western blot for this purpose.
We would like to extend the therapeutic approach with pharmacological chaperones to other pathologies that have no cure at present.
Phosphomannomutase 2 deficiency represents the most frequent type of congenital disorders of glycosylation. The complete loss of phosphomannomutase activity is probably not compatible with life and people affected carry at least one allele with residual activity. We characterized wild type phosphomannomutase 2 and its most common hypomorphic mutant, p.F119L which is associated with a severe phenotype of the disease. We demonstrated that active species is the dimeric enzyme and that the mutation weakens the quaternary structure and, at the same time, affects the activity and the stability of the enzyme. We demonstrated that ligand binding stabilizes both proteins, wild type and F119L-phosphomammomutase 2 and promotes subunit association in vitro. The strongest effects are observed with glucose-1,6-bisphosphate or with monophosphate glucose in the presence of vanadate. This finding offers a novel approach for the treatment of phosphomannomutase 2 deficiency.

ABSTRACT N. 84

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**ANDRIA GENEROSO**	
Telethon grant N.	**GUP09017**	
Total budget €	**260.000**	
Centres: **4**	*Duration (yrs):* **1**	*Starting year:* **2011**

COMBINED ENZYME ENHANCEMENT THERAPY (EET) AND ENZYME REPLACEMENT THERAPY (ERT) IN PATIENTS WITH POMPE DISEASE

Parenti Giancarlo (1,2), la Marca Giancarlo (3), Fecarotta Simona (1), Donati Maria Alice (4), Morandi Lucia Ovidia (5), Ravaglia Sabrina (6), Moglia Arrigo (6), Ascione Serena (1), Ombrone Daniela (3), Sacchini Michele (4), Pasanisi Barbara (5), De Filippi Paola (7), Rosa Margherita (1), Agovino Teresa (1,4), Porto Caterina (1,2), Rossi Barbara (2), Carbone Maria Teresa (8), Costantino Demetrio (9), Concolino Daniela (10), Andria Generoso (1)

(1) Dipartimento di Pediatria, Università di Napoli "Federico II", Via S. Pansini 5, 80131, Napoli, Tel. +39.081.7462673, Fax +39.081.7463116; E-mail: andria@unina.it
(2) Telethon Institute of Genetics and Medicine, Napoli
(3) Dipartimento di Farmacologia Clinica, Ospedale Pediatrico Meyer, Firenze
(4) UO Malattie Metaboliche e Muscolari Ereditarie, Ospedale Pediatrico Meyer, Firenze
(5) UO Patologia Muscolare-Neuroimmunologia, Fondazione IRCCS Istituto Neurologico Besta, Milano
(6) Dipartimento di Sanità Pubblica e Neuroscienze, Università di Pavia
(7) Dipartimento di Medicina Molecolare, Università di Pavia
(8) Ospedale SS Annunziata, AORN Santobono-Pausilipon, Napoli
(9) AO Bianchi Melacrino Morelli, Reggio Calabria
(10) Pediatria, Università "Magna Graecia" di Catanzaro

Background. Pompe disease (PD) or Glycogen storage disease type II (OMIM 232300) is a metabolic myopathy, with a wide phenotypic variability, due to the deficiency of the lysosomal hydrolase alpha-glucosidase (acid maltase, GAA). Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) is the only approved treatment for PD patients, but its efficacy is variable, particularly on the skeletal myopathy. Our recent studies performed in vitro and in an animal model of PD (Porto et al. Mol Ther. 2009;17: 964-71) showed that a pharmacological chaperone, miglustat (NB-DNJ) enhances the activity of rhGAA.
Aim. To evaluate whether the combined treatment with ERT and NB-DNJ is more effective in correcting the enzyme defect in PD patients.
Patients. We enrolled 13 PD patients (age range: 5-52 yrs) with various clinical phenotypes: 1 classic infantile form (CI), 2 non-classic infantile form (NCI) and 10 late onset form (LO).
Primary end-points. Increased blood GAA activity at different times after the administration of ERT alone or ERT in combination with NB-DNJ (approx. 80 mg/m2 on the evening before infusion and 80-100 mg/m2 t.i.d. on the day of infusion); decreased plasma creatine kinase (CK) levels, before and after the start of the combination treatment.
Methods. GAA activity was assayed by tandem mass spectrometry in dried blood spots taken 24 hrs after ERT (T1) and successively every 48 hrs for 14 days until the next ERT. Specimens were collected in 3 cycles of ERT without NB-DNJ and 3 cycles of ERT with NB-DNJ, respectively. Statistical analysis was performed by Mann-Whitney test and/or Student-t test, if applicable.
Results. The trial is still ongoing. The preliminary data so far obtained show that in each patient GAA activity at T1 during the combination treatment was higher than the activity with ERT alone. In the 13 patients the increase in activity ranged between 1.6- and 63.1-fold and it was statistically significant ($P<0.05$) in 11 patients (1 CI, 1 NCI and 9 LO). The mean GAA activity at T1 of the whole patients population during the combined treatment was 6,9 times higher than that during ERT alone ($P<0.00001$). Plasma CK levels were comparable between the cycle with ERT alone and the cycle with combined treatment.
Conclusion. The results of this study, though still preliminary, indicate that the combined therapeutic approach can enhance in vivo the activity of the rhGAA and suggest that a treatment protocol based on this approach may translate into more effective correction of the enzymatic defect in PD. Our data seem particularly encouraging for the LO patients, in whom ERT show a variable and limited efficacy on the skeletal myopathy. The same combination of ERT and specific pharmacological chaperones might be applied to other lysosomal storage disorders, such as Fabry disease (Porto et al, J Inherit Metab Dis. 2012 ;35:513-20).

ABSTRACT N. 85

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CARRA SERENA**	
Telethon grant N.	**GEP12008**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

CHARACTERIZATION OF THE R7S MUTATION OF HEAT SHOCK PROTEIN HSPB3 AND OF TWO NOVEL MUTATIONS FOUND IN PATIENTS SUFFERING OF CONGENITAL MYOPATHY: UNDERSTANDING THE MECHANISMS LEADING TO DISEASE

Heldens Lonneke (1), Verbeek Dineke (2), Angelini Corrado (3), Boelens Wilbert (4), Tupler Rossella (5), Carra Serena (1)

(1) Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Università degli Studi di Modena e Reggio Emilia, via Giuseppe Campi, 287, 41125 Modena, Italia, Tel. +39.059.2055421 Fax +39.059.2055363 E-mail: serena.carra@unimore.it
(2) Centro Medico Ospedaliero di Groningen, Università degli Studi di Groningen, Dipartimento di Genetica, 9700 RB Groningen, Paesi Bassi
(3) Dipartimento di Neuroscienze, Università degli Studi di Padova, Padova, Italia
(4) Radboud Università degli Studi di Nijmegen, Nijmegen, Paesi Bassi
(5) Dipartimento di Scienze della Vita, Università degli Studi di Modena e Reggio Emilia, Modena, Italia

HSPB3 is a poorly characterized member of the small HSP/HSPB family (HSPB1-HSPB10) that forms a complex with HSPB2 with a defined 1:3 ratio. The HSPB2/HSPB3 complex is induced during muscle differentiation and plays a role in muscle maintenance. Recently the R7S mutation in HSPB3 has been associated with distal hereditary motor neuropathy type 2C (dHMN 2C). Here we report the identification in myopathic patients of two novel mutations in HSPB3: 1) one mutation affects the R116 residue, which corresponds to a key amino acid in the alpha-crystallin domain, whose mutation in other members of the HSPB family also causes disease (it is equivalent to e.g. R120 in HSPB5, whose mutation into G causes MFM and to K141 in HSPB8, whose mutation into E or N causes dHMN); 2) the other mutation disrupts the reading frame leading to a premature stop codon at amino acid 50. Both mutations were not found in more than 400 normal alleles. Expression studies allowed us to confirm that the mutation causing a premature stop codon leads to the generation of an unstable protein that is likely immediately degraded after synthesis and cannot be detected. Also, while both expressed, the R7S mutant was more stable than the R116 one. We next characterized in cells and in vitro the ability of these HSPB3 mutants to interact with HSPB2 and form the HSPB2/HSPB3 complex. We found that while the R7S mutant of HSPB3 was still able to interact with HSPB2, the R116 mutant was not. Future studies will allow us to better characterize how these HSPB3 mutants affect HSPB3 and, indirectly, HSPB2 stability, subcellular localization and function. They will also elucidate on HSPB3 and HSPB2 function in both motor neurons and myoblasts and will shed light on how mechanistically the mutations in HSPB3 affect the function and viability of these cell types, contributing to disease.

ABSTRACT N. 86

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**DI BLASI CLAUDIA**	
Telethon grant N.	**GEP12074**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

ASSESEMENT OF THE PATHOGENIC ROLE OF A MISSENSE VARIANT IN A BENIGN AUTOSOMAL DOMINANT MYOPATHY WITH HYPERCKAEMIA

D'Adamo Maria Cristina (2), Saredi Simona (1), Vasso Michele (3), Morandi Lucia (1), Gelfi Cecilia (3), Pessia Mauro (2), Mora Marina (1), Di Blasi Claudia (1)

(1) Division of Neuromuscular Diseases and Neuroimmunology, Foundation, Neurological Institute C. Besta, Via Temolo 4, 20126 Milano, Tel. 02.23942625, Fax 02.23942619, E-mail: cdiblasi@istituto-besta.it
(2) Section of Human Physiology, University of Perugia School of Medicine, Perugia
(3) Department of Biomedical Sciences for Health, University of Milan, Segrate, Milan

Chronic elevation of plasma CK levels is a common manifestation of neuromuscular disorders and may precede clinical expression of the disease. Persistent high CK is frequently encountered in asymptomatic patients with normal neurological examination and with a benign course. Malignant hyperthermia (MH), a life-threatening condition that is usually triggered by exposure to drugs used for general anaesthesia, may present with hyperCKaemia as only clinical sign. Recently, we observed 7 patients from 3 unrelated Italian families, with hyperCKaemia and a benign myopathy in whom the muscle biopsy showed mild pathological findings and calsequestrin 1 (CSQ1)-positive sarcoplasmic inclusions in many fibers. In these patients we performed molecular analysis of the CASQ1 gene, that encodes CSQ1, and found a heterozygous missense mutation in a evolutionarily conserved residue. Linkage analysis indicated an identical mutation associated haplotype, suggesting remote consanguinity and a founder effect.
CSQ is the main sarcoplasmic reticulum calcium binding and storage protein localized in the terminal cisternae of skeletal and cardiac muscle cells. Two CSQ isoforms, encoded by the CASQ1 and CASQ2 genes, are expressed in skeletal muscle and heart and have dissimilar calcium binding capacities. Mutations in CASQ2 lead to severe functional changes including ventricular arrhythmias and sudden cardiac death, while the CASQ1 gene has never been linked to human diseases. However, in mice, CASQ1 genetic ablation causes an increased susceptibility to hypermetabolic syndrome in response to halothane- and heat-exposure, similar to human MH.
CASQ1 mutations could affect calcium storage and release, CSQ1 polymerization and folding, leading to the aggregation of the protein, causing defects in muscle contractile function and severe damage to the muscle cell.
Specific aims of the project are: (1) validation of the pathogenic effect of the mutationt on CSQ1 expression; (2) evaluation of CSQ1 protein modifications, by means of proteomic analysis; (3) assessment of the effect of the mutation on Ca++ homeostasis.
(1) Transient transfection of COS-7 cells using wild-type and mutant constructs, showed that the wild-type protein was uniformly distributed as punctate inclusions throughout the cytoplasm while the mutated protein formed abnormal large cytoplasmic aggregates, indicating that the mutation is pathologically relevant. Stable transfection of C2C12 muscle cells are ongoing. (2) Proteomic analysis on protein extracts from patient and control muscle cells is being performed. (3) Xenopus laevis oocytes, a model system extensively used for studying calcium signaling, have been micro-injected with wild-type and mutant mRNAs for electrophysiological studies and live imaging analyses.
This study will provide important information both at clinical and basic science level on CSQ1 role in muscle contraction in health and disease.

ABSTRACT N. 87

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**VAZZA GIOVANNI**	
Telethon grant N.	**GEP12083**	
Total budget €	**43.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

IDENTIFICATION OF THE GENE RESPONSIBLE FOR A NEW FORM OF DISTAL MYOPATHY

Boaretto Francesca (1), Salvoro Cecilia (1), Pegoraro Elena (2), Mostacciuolo Maria Luisa (1), Giovanni Vazza (1)

(1) Dipartimento di Biologia, Università di Padova - Via G. Colombo 3, 35131 Padova; Tel. 049 8276226, Fax 049 8276209, E-mail: giovanni.vazza@unipd.it
(2) Dipartimento di Neuroscienze, Università di Padova

Distal myopathies (DM) belong to a larger group of rare progressive genetic disorders characterized by atrophy and weakness of the voluntary distal muscles of the upper and lower limbs.
By combining clinical and molecular findings, more than 20 different genetic entities have been reported to date. For 14 of them, the causative gene has been identified, while only the genetic localization is known for the remaining forms.
The aim of this project is to identify the gene responsible for a new form of distal myopathy segregating as an autosomal dominant trait in a large Italian family. Patients show an early onset of the disease with a predominant involvement of hand muscles.
As first, we evaluated the possible involvement of genes known to be responsible for distal myopathies with similar clinical features to those observed in our family. Direct sequencing excluded mutations in the KLHL9 (MIM 611201), and Cav3 (MIM 601253) genes. The

other dHMN forms, including Welander locus (MIM 604454), MYH7 (MIM160500), Myotilin (MIM609200), Matrin 3 (MIM606070) and Filamin C (MIM614065) were evaluated by indirect linkage analysis with strictly associated microsatellite markers.
Subsequently, we performed a genome-wide linkage analysis using high density SNP-array and identified 3 regions co-segregating with the disease in all affected subjects (chromosomes 1, 13 and 17). Again, none of these regions overlaps to known distal myopathy loci or other genes/loci associated to similar muscular phenotypes, thus supporting the existence of a new DM gene. These three candidate regions span 9 megabases and contains more than 150 known genes.
In order to achieve the final objective, we plan to combine our preliminary results with a next-generation sequencing approach. At present, whole exome enrichment and sequencing are in progress for three subjects of our family. Once completed this step, an analytical pipeline for high confidence variant calling, annotation and prioritization will be adopted in order to look for pathogenic mutations within the three candidate regions. Putative disease-causing mutations will be then validated by confirming their segregation within the family and by excluding their presence in a matched control sample. Furthermore, the discovered disease-gene will be screened in a cohort of patients affected by distal myopathy with unknown genetic aetiology.

ABSTRACT N. 88

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BRUNO CLAUDIO**	
Telethon grant N.	**GUP08005**	
Total budget €	**169.000**	
Centres: **9**	*Duration (yrs):* **2**	*Starting year:* **2009**

CLINICAL, MORPHOLOGICAL AND MOLECULAR STUDY OF ITALIAN PATIENTS WITH CONGENITAL MYOPATHY

Cassandrini Denise (1), Berardinelli Angela (2), Bertini Enrico (3), Comi Giacomo P. (4), Mercuri Eugenio (5), Mongini Tiziana (6), Nigro Vincenzo (7), Pegoraro Elena (8), Toscano Antonio (9), Mora Marina (10), Morandi Lucia (10), Battini Roberta (1), D'Amico Adele (3), Fattori Fabiana (3), Magri Francesca (4), Pane Marika (5), Codemo Valentina (8), Messina Sonia (9), Santorelli Filippo M. (1), Bruno Claudio (11)

(1) U.O. Medicina Molecolare, IRCCS Fondazione Stella Maris, Calambrone, Pisa, Italy
(2) Dipartimento di Neuropsichiatria Infantile, IRCCS Fondazione Mondino, Pavia, Italy
(3) U.O. Medicina Molecolare, IRCCS Ospedale Bambino Gesù, Roma, Italy
(4) Dipartimento di Scienze Neurologiche, Fondazione Ca' Granda, Univ. degli Studi di Milano, Milano, Italy
(5) Dipartimento di Neuropsichiatria Infantile, Policlinico A. Gemelli, Univ. Cattolica Sacro Cuore, Roma, Italy
(6) Dipartimento di Neuroscienze, Univ. degli Studi di Torino, Torino, Italy
(7) Centro Interdipartimentale di Ricerca, Seconda Univ. degli Studi di Napoli, Napoli, Italy
(8) Dipartimento di Neuroscienze, Univ. degli Studi di Padova, Padova, Italy
(9) Dipartimento di Neuroscienze, Univ. degli Studi di Messina, Messina, Italy
(10) U.O. Malattie Neuromuscolari, IRCCS Istituto C. Besta, Milano, Italy
(11) U.O. Neurologia Pediatrica e Malattie Muscolari, IRCCS Istituto Giannina Gaslini, Largo G. Gaslini 5, 16147 Genova, Italy, Tel. +39.010.5636756, Fax +39.010.3538265, E-mail: claudiobruno@ospedale-gaslini.ge.it

Background
The congenital myopathies (CM) encompass a group of neuromuscular disorders with onset of muscle weakness at birth and can be classified on the basis of distinctive morphological abnormalities in skeletal muscle. Advances in molecular genetic research have brought to new genetic-morphological correlations increasing the number of clinical-genetic entities and providing new insight into their pathogenesis. However, most of the congenital myopathies are genetically heterogeneous and the genotype-phenotype relationship is not well defined yet with important implication for genetic counselling. In addition many patient remain without genetic signature making probable that further new genes have to be found.
Aim of this project
This is an open multicentre collaborative study, initially involving nine Italian Centers for Neuromuscular Diseases, with the global aim to collect detailed clinical, morphological, and genetic data in a large group of patients with congenital myopathy. In the first year, extensive review of clinical and morphological data allowed to recognize a total of 275 patients. Patients were classified according to morphological features as proposed by North (Neuromuscul Disord 2008; 18:433-442), in four subgroups: Nemaline Myopathy (NM) (52 patients, 19%), Myopathy with Cores (MC) (133 patients, 48%), Myopathy with central nuclei (MCN) (33 patients, 12), and Myopathy with fibre type disproportion (CFTD) (57 patients, 20%). In the following years extensive genetic testing was performed by Sangers analysis of the following genes: ACTA1, TPM2, TPM3, RYR1, SEPN1, MTM1, DNM2, MYH2, MYH7. Prioritisation of the most appropriate gene to test in each patient was based on the relative frequency of that specific gene in that particular subgroup of CM. In particular in NM patients ACTA1, TPM2, TPM3.
Results
Out of the 52 NM patients, 12 had mutations in the ACTA1 whereas no mutations have been identified in TPM2 or TPM3 genes. NEB1 mutations have been identified in 7 out of 8 patients throughout a targeted deep sequencing analysis. The analysis of this latter gene is still ongoing. Among the 133 MC patients, 58 had mutations in RYR1 gene, and 5 in SEPN1 (Cagliani et al, J Neurol Sci 2011). Regarding RYR1 mutations, recessive variants were at least as frequent as the dominant ones. The first were distributed throughout the entire coding sequence (2/3 in hot spot regions), whereas around 84% of dominant mutations were typically missense in hot spot regions. In 33 patients with MCN, 9 patients carried MTM1 and 10 DNM1 mutations (Catteruccia et al, in press). Four mutations (1 ACTA1, 2 TPM3, and 1 TPM3) were identified in CFTD.
Conclusion
We have studied a large cohort of Italian patients with CM, being the RYR1-related myopathies by far the most common cause of congenital myopathies in Italy (Bruno et al. Acta Myologica 2012). We have confirmed a marked variability of clinical presentation and histopathologic findings, as well as different modes of inheritance. When performed, muscle MRI showed distinctive pattern of muscle involvement, resulting to be an important diagnostic tool to guide the clinician for appropriate genetic analysis. The overall detection mutations rate is about ~41%. However a large cohort of DNA samples are being processed by whole exome sequencing. Extensive analysis of data are in progress in order to establish whether the combination of information available from the individual findings can facilitate the diagnostic challenge of selecting the most appropriate genetic test.

ABSTRACT N. 89

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BERNARDI PAOLO**	
Telethon grant N.	**GGP11082**	
Total budget €	**730.000**	
Centres: **5**	*Duration (yrs):* **2**	*Starting year:* **2011**

TOWARD A MITOCHONDRIAL THERAPY OF COLLAGEN VI MUSCULAR DYSTROPHIES

Bonaldo Paolo (1), Rizzuto Rosario (1), Maraldi Nadir M (2,3), Gelfi Cecilia (4), Argenton Francesco (5), Di Lisa Fabio (1), Merlini Luciano (3), Reggiani Carlo (1), Sabatelli Patrizia (3), Bernardi Paolo (1)

(1) Dipartimento di Scienze Biomediche, Università di Padova, Viale Giuseppe Colombo 3, 35131 Padova, Tel. 049.8276365, Fax 049.8276361, E-mail: bernardi@bio.unipd.it
(2) Dipartimento di Scienze Anatomiche, Università di Bologna
(3) Laboratorio di Biologia Cellulare Muscoloscheletrica, Istituto Ortopedico Rizzoli, Bologna
(4) Dipartimento di Bioimmagini e Fisiologia Molecolare, Consiglio Nazionale delle Ricerche, Milano
(5) Dipartimento di Biologia, Università di Padova

Objective of this program is to get a detailed mechanistic understanding of the pathogenesis of Collagen (Col) VI muscular dystrophies, with specific emphasis on the role of mitochondrial dysfunction. Our goal is to translate the advances made in the animal models into effective treatments for the human diseases. Specific aims of this project are to (i) assess the relative role of mono amine oxidases (MAO) overactivation, Ca2+ deregulation and defective organelle clearance in causing/amplifying mitochondrial dysfunction through the permeability transition pore (PTP), and its consequences on energy metabolism; (ii) identify the adaptive mechanisms that in muscle-derived cells from patients spontaneously attenuate mitochondrial dysfunction during culture; (iii) define the molecular basis for impaired muscle regeneration/differentiation and their contribution to ColVI disease pathogenesis; and (iv) develop a combinatorial therapy aimed at these multiple targets in preclinical models of ColVI diseases. The rationale rests on our discoveries that (i) mitochondrial dysfunction mediated by inappropri-

ate opening of the PTP plays a key role in ColVI myopathies, and that Col6a1-/- mice can be cured by cyclosporin A, which also gave encouraging results in a pilot trial; and (ii) impaired removal of defective mitochondria amplifies the defect, which is worsened by excessive oxidative stress and Ca2+ accumulation in the mitochondria. We will assess the efficacy of PTP-active derivatives of cyclosporin A that do not cause immunosuppression but are effective in the mouse model and in patients' cells, and test novel treatments based on the targets mentioned above (PTP, MAO, Ca2+ homeostasis, autophagy). The anticipated output is defining and validating a combinatorial therapy effective in the treatment of human ColVI muscular dystrophies.

ABSTRACT N. 90

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**MERLINI LUCIANO**	
Telethon grant N.	**GUP11007**	
Total budget €	**77.300**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

LOW-PROTEIN DIET TO CORRECT DEFECTIVE AUTOPHAGY IN PATIENTS WITH COLLAGEN VI RELATED MYOPATHIES

Battistini Nino Carlo (2), Bernardi Paolo (3), Bonaldo Paolo (4), Cocchi Daniela (5), Ripamonti Claudio (6), Sabatelli Patrizia (8), Ferlini Alessandra (7), Merlini Luciano (1)

(1) Laboratorio di Biologia Cellulare Muscoloscheletrica, Istituto Ortopedico Rizzoli, via di Barbiano 1/10, 40136 Bologna, Tel. 051.6366779, Fax 051.583593, E-mail: luciano.merlini@ior.it
(2) Cattedra di Scienze Tecniche Dietetiche Applicate, Università di Modena
(3) Dipartimento di Scienze Biomediche Sperimentali, Università di Padova
(4) Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova
(5) Dipartimento di Scienze Statistiche Paolo Fortunati, Università di Bologna
(6) Medicina, Istituto Ortopedico Rizzoli, Bologna
(7) Dipartimento di Medicina Sperimentale e Diagnostica, Genetica Medica, Università di Ferrara
(8) CNR, Istituto di Genetica Molecolare-IOR, Bologna

The study includes a 3-month observational phase on the natural course, followed by a 12-month, open label, non-comparative, single arm, phase II pilot study on the efficacy, safety and tolerability of a low protein diet (LPD) in 8 adult patients with Bethlem Myopathy (BM) and Ullrich Congenital Muscular Dystrophy (UCMD).
The study has been registered: http://clinicaltrials.gov Identifier (NCT01438788)
Results of the observational phase
We studied the relationship between body composition, muscle strength, exercise capacity and pulmonary function in adult patients with BM and UCMD due to collagen type VI (COL6) mutations. Fat free mass (FFM) and percentage fat mass (%FM) was assessed by dual-energy X-ray absorptiometry (DXA), isometric muscle strength by a dynamometer, exercise capacity by the distance walked in 6 min (6MWD) and pulmonary function with a spirometer. In spite of a BMI below 30 (mean +/- SD: 22.9 +/- 3.7%) COL6 subjects showed a marked increase regional fat tissue mass (mean +/- SD: 45.3 +/- 12.6%). Muscle strength was diffusely reduced particularly elbow flexion (mean +/- SD: 63 +/- 12 N) and knee extension (mean +/- SD: 98 +/- 22 N). There was a good correlation between muscle strength and lean tissue mass (r=0.84), %FVC (r=0.78), and the 6MWD (r=0.74). There was however no correlation between BMI and muscle strength (r=-0.03), 6MWD (r=-0.10), or %FVC (-0.002). A good correlation was evident between 6MWD and %FVC (r=0.73).
Conclusions:
i) COL6 subjects have high regional fat tissue mass despite normal BMI.
ii) Regional lean tissue mass correlates with residual strength, exercise capacity and pulmonary function.
We compared the ability of bioelectric impedance analysis (BIA) and skinfold thickness (ST) measurements to estimate changes in body composition in 7 adult patients with BM. We assessed fat free mass (FFM) and percentage fat mass (%FM) by BIA, ST and a criterion method, dual-energy X-ray absorptiometry (DEXA). When compared with DEXA (mean +/- SD: 34.2 +/- 8.6), ST measurements (46.8 +/- 8.3 kg) and BIA (43.5 +/- 8.6 kg) significantly (P<0.0001) overestimated FFM, which led to an underestimation of the percentage of fat mass (ST: 27.0 +/- 6.3%; BIA: 31.8 +/- 11.5%; DEXA: 44.1+/- 13.2%). In contrast, estimates obtained by ST and BIA were not significantly different (P=0.16). DEXA defined all the patients as obese, whereas body mass index was below 30 in all patients and ST measurements defined 2 and BIA 3 patients as obese.
Conclusion:
Body composition estimates by ST and BIA are not adequate to detect fat accumulation in patients with BM.
Patients leukocytes have been isolated from blood samples collected at day -85, -45 and 1. ELISA and western blot analysis, beclin1, BNIP3 and LC3-II values were within a range of normality with a minimal inter and intra-individual variability (as compared with 5 healthy subjects, 21-52 years old); only in one BM patient we found a mild reduction of LC3-II lipidated protein. The analysis of autophagy markers in the patients muscle biopsies obtained at day 1, is in progress. Myoblasts, skin fibroblasts and melanocytes cultures have been also established for biochemical, morphological and functional studies.
Update of the LPD pilot study
November 2011 and February 2012: recruitment of the 8 patients who entered in the observational phase.
February and April 2012: termination of the observational phase and inclusion in the LPD trial.

ABSTRACT N. 91

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SZABADKAI GYORGY**	
Telethon grant N.	**GEP12066**	
Total budget €	**49.500**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

THE ROLE OF INOSITOL 1,4,5-TRISPHOSPHATE MEDIATED NUCLEAR CA2+ SIGNALS IN CORE MYOPATHIES

Szabadkai Gyorgy, Suman Matteo

Dipartimento di Scienze Biomediche, Complesso Interdipartimentale Vallisneri, Universita degli Studi di Padova, Viale Colombo, 3 - Via Marzolo, 3 35131 Padova, Italy, Tel. +39 049 827 6047; E-mail: gyorgy.szabadkai@unipd.it

Disruption of cellular Ca2+ homeostasis underlies most muscle pathologies. It is particularly apparent in core myopathies, caused by mutations in the ryanodine receptor Ca2+ release channel, such as Central Core Disease (CCD) and Multi Minicore Disease (MmD). However, several lines of evidence indicate that depolarizing stimuli induce two independent cellular Ca2+ signals in muscle fibers: a fast signal associated with excitation-contraction coupling and a slow signal that is preferentially formed in the nucleus and regulates gene expression. While the role of RyR1 mutations in the disruption of EC coupling has been extensively studied, the role of the nuclear Ca2+ dependent pathways remains completely unexplored in these diseases.
We have recently described a signalling complex formed by plasmamembrane receptors coupled to inositol 1,4,5-trisphosphate (IP3) production in the T-tubules associated with nuclear IP3 receptors (IP3R), responsible for local control of Ca2+ mediated nuclear gene transcription in cardiomyocytes. In addition, our preliminary evidence indicates that the cellular distribution and expression of IP3Rs is altered in cellular and animal models of CCD. Based on these premises we propose that (i) similarly to the cardiac muscle, IP3R mediated local nuclear Ca2+ signalling controls gene expression in skeletal muscle, and (ii) the phenotype of CCD can be the result of disruption of the IP3 mediated nuclear Ca2+ signalling and consequent alterations in gene expression.
Here we will show our first set of data to morphologically and functionally characterize the nuclear IP3/Ca2+ signal generating system at the interface between the T-tubules and the nuclear envelope using molecular biology, genetic and cellular imaging approaches. Our final aim is to determine the role of the IP3 mediated nuclear Ca2+ signalling in transcriptional regulation in normal and CCD muscle, leading to potentially novel strategies in treatment of the disease.

ABSTRACT N. 92

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PROTASI FELICIANO**	
Telethon grant N.	**GGP08153**	
Total budget €	**484.900**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2008**

CALSEQUESTRINS IN CALCIUM HOMEOSTASIS AND POTENTIAL ROLE IN INHERITED HUMAN SKELETAL MUSCLE DISEASES

Michelucci Antonio (1), Paolini Cecilia (1), Pietrangelo Laura (1), Dirksen Robert T. (2), Reggiani Carlo (3), Protasi Feliciano (1)

(1) CeSI - Centro Scienze dell'Invecchiamento & DNI - Dipartimento di Neuroscienze ed Imaging; Università G. d'Annunzio, via Colle dall'Ara, 66100, Chieti, Italy, Tel. 0871.541423; Fax 0871.541422, E-mail: fprotasi@unich.it
(2) Department of Pharmacology and Physiology, University of Rochester Medical Center, NY, USA
(3) Dipartimento di Anatomia e Fisiologia, Università di Padova

Background. To date, Malignant Hyperthermia (MH) is only seen as a clinical syndrome in which genetically predisposed individuals respond to volatile anesthetics in the operating room with life-threatening episodes characterized by elevations in body temperature and rhabdomyolysis of muscle fibers. However, similar over-heating episodes - known as heat strokes (HS) - have been reported in individuals also after exposure to environmental heat and/or physical exertion. Several important issues still need to be resolved: a) mutations in RYR1, the sarcoplasmic reticulum (SR) Ca2+ release channel, have been found in many, but not all, MH cases suggesting the involvement of additional genes; b) the relationship between MH and HS is not yet widely recognized; c) the molecular mechanisms leading to rhabdomyolysis of skeletal fibers are not fully understood.
Results. Thanks to Telethon ONLUS support (GGP08153), we have demonstrated in animal models that: a) MH/HS episodes can result not only from mutations in RYR1, but also from mutations in proteins that modulate RYR1 function, like Calsequestrin-1 (CASQ1); b) the molecular mechanisms underlying hyperthermic episodes triggered by anesthetics and heat are virtually identical and involve: i) oxidative stress-mediated RYR1 Ca2+ leak, ii) severe SR depletion, and iv) Ca2+ influx from extracellular space due to activation of store operated Ca2+ entry (SOCE). To prove our hypothesis, we have successfully cured curing CASQ1-null mice suffering of MH and HS by treatment for 2 months with N-acetylcysteine (NAC, a potent anti-oxidant): the rate of mortality following exposure to halothane (2%, 1h at 32°C) and heat (41°C, 1h) was respectively 78.6 vs 25.0% and 85.7 vs 30.0% in controls vs NAC-treated animals. This protection is likely mediated by: a) reduction of oxidative stress, as shown by a decrease of mitochondrial superoxide flashes (mSOF) frequency ($P<0.05$); b) decreased internal temperature during heat-stress protocol (from 42.1 to 40.8 °C); and c) lower number of fibers undergoing rhabdomyolysis (from 37.6 to 11.6 %). NAC treatment was also effective in increasing the threshold for caffeine-induced contracture (in-vitro contracture test or IVCT). These results suggest that a) increased oxidative stress exacerbates the RYR1 instability that underlies MH/HS episodes and b) anti-oxidants should be considered for the prevention/treatment of over-heating skeletal muscle disorders.
Perspectives. Considering high frequency and life-threatening nature of these syndromes, there is a critical need to develop new/effective drugs for their treatment. In the last 3 years we have moved significant steps toward this goal. Since abnormal molecular mechanisms are potential targets for prevention and treatment, the current goal is to develop/test new compounds capable of reducing Ca2+ leak, diminishing oxidative stress, and blocking SOCE in muscle fibers.

ABSTRACT N. 93

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PENNUTO MARIA**	
Telethon grant N.	**GGP10037**	
Total budget €	**190.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

INSULIN-LIKE GROWTH FACTOR 1/AKT AND ANDROGEN SIGNALING CROSSTALK IN THE PATHOGENESIS OF SPINAL AND BULBAR MUSCULAR ATROPHY

Scaramuzzino Chiara (1), Milioto Carmelo (1), Casci Ian (2), Fackelmeyer Frank (3), Pandey Udai (2), Pennuto Maria (1)

(1) Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genoa, Italy, Tel. +39.010.71781793, Fax +39.010.71781230
(2) Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA, USA
(3) Biomedical Research Institute (BRI-FORTH), Ioannina, Greece

Spinal and bulbar muscular atrophy (SBMA), also named Kennedy's disease, is a rare genetic motor neuron disease that manifests only in males. SBMA is characterized by the dysfunction and loss of lower motor neurons from brainstem and spinal cord and skeletal muscle atrophy. SBMA is caused by expansion of a polyglutamine (polyQ) tract in the gene coding for androgen receptor (AR). AR is a transcription factor activated by testosterone. The gender-specificity of SBMA is the result of the interaction of mutant AR with its natural ligand, testosterone.
We have previously shown that a specific post-translational modification of mutant AR, i.e. phosphorylation by the kinase Akt, blocks the binding of AR to testosterone, thereby preventing toxicity (Palazzolo et al., 2007). Moreover, we showed that activation of Akt by overexpression of the insulin-like growth factor 1 (IGF-1) selectively in the skeletal muscle attenuates disease progression and manifestations in a mouse model of SBMA (Palazzolo et al., 2009). Based on these data, we proposed to investigate how phosphorylation of polyQ-AR is regulated at the post-translational level.
Akt phosphorylates mutant AR at serine 215 and 792, which lie in the Akt consensus site RxRxxS (where R is arginine, X any amino acid, and S serine). Arginine residues can be modifies at the post-translational level by methylation. We are testing the hypothesis that methylation of the R residues at the Akt consensus site affects the phosphorylation of mutant AR. Arginine methylation is operated by a family of enzymes, the protein arginine methyltransferases (PRMTs). Mammalian cells express at least eight PRMTs, PRMT1, 2, 3, 4, 5, 6, 7, and 8. We provide evidence for the first time to our knowledge that both normal AR and mutant AR co-localizes and form a complex with PRMT2, PRMT6, and PRMT7 in cultured cells. Importantly, we show that PRMT6, but not the other PRMTs, specifically transactivates normal and mutant AR. Moreover, we found that PRMT6 requires the R residues at the Akt consensus site to fully transactive the AR. To assess whether this functional interaction is pathogenetic, we crossed SBMA flies with flies in which PRMT6 was knock down. Preliminary evidence shows that PRMT6 knock down attenuates the degeneration induced by mutant AR in the fly eye. In conclusion, we report here the identification of PRMT6 as a novel mutant AR co-factor, whose interaction with mutant AR is pathogenetic, highlighting the contribution of alteration of native AR function in SBMA pathogenesis.

ABSTRACT N. 94

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**COMI GIACOMO PIETRO**	
Telethon grant N.	**GGP10062**	
Total budget €	**399.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

DEVELOPMENT OF A THERAPEUTIC APPROACH FOR SPINAL MUSCULAR ATROPHY WITH RESPIRATORY DISTRESS (SMARD1) USING HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL STEM CELLS AND MOTOR NEURONS

Nizzardo Monica, Simone Chiara, Rizzo Federica, Ruggieri Margherita, Salani Sabrina, Brajkovic Simona, Bresolin Nereo, Corti Stefania, Comi Giacomo

Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy, Tel. +39.02.55033817, Fax +39.02.55033800; E-mail:giacomo.comi@unimi.it

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is a fatal form of infantile motor neuron disease caused by mutations in the IGHMBP2 gene. Currently, there is no cure. We previously described that transplantation of motor neurons derived from murine pluripotent stem cells can ameliorate the disease phenotype in a SMARD1 mouse model.
In this project, we investigated the therapeutic potential of transplantation of human neural stem cells and motor neurons derived from induced pluripotent stem cells (iPSCs) as therapeutic strategy for SMARD1. We generated iPSCs from human healthy fibroblasts, using a non viral method based on non integrating episomal vectors. These cells were differentiated into neural stem cells and motor neurons and characterized by morphological, gene expression, and protein analysis. iPSC-purified motoneurons were transplanted into the spinal cords of SMARD1 mice. We determined the survival, differentiation, and function of motor neurons after engraftment. We identified human-derived motoneurons, which presented motoneuronal phenotype and coexpressed HB9 and ChAT, within the ventral horns of all transplanted animals. We demonstrated also that transplanted cells improve the phenotype of treated animals in their neuromuscular function and survival. Our goal is to examine

bi-directional cellular and molecular interactions between transplanted donor-derived motor neurons and host cells to elucidate the mechanisms that underlie the amelioration of disease phenotype other than cell replacement. This study demonstrated that transplantation of iPSC derived motoneurons is feasible in an animal model of a human motoneuron disease, contributing in the development of a therapy for SMARD1 and other motor neuron disorders.

ABSTRACT N. 95

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CORTI STEFANIA**	
Telethon grant N.	**GGP09107**	
Total budget €	**257.900**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

NEUROPROTECTION IN SPINAL MUSCULAR ATROPHY (SMA) USING NEURAL STEM CELLS AS A THERAPEUTIC APPROACH

Simone Chiara (1), Nizzardo Monica (1), Ronchi Dario (1), Salani Sabrina (1), Riboldi Giulietta (1), Magri Francesca (1,2), Faravelli Irene (1), Zanetta Chiara (1), Menozzi Giorgia (2), Bonaglia Chiara (2), Bresolin Nereo (1,2), Comi Giacomo (1), Corti Stefania (1)

(1) Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy, Tel. 39.02.5503807, Fax +39.02.55033800; E-mail: stefania.corti@unimi.it
(2) IRCCS E. Medea La Nostra Famiglia, Bosisio Parini, LC

Spinal muscular atrophy (SMA) is a genetic motor neuron disease caused by mutations in the SMN1 gene. SMA is among the most common genetic neurological diseases causing infant mortality without any effective cure. In this project we explored the therapeutic potential of human NSCs (hNSCs) and motor neurons derived from induced pluripotent stem cells (iPSCs) as a tool for cell mediated therapy for SMA. Indeed, the use of patient autologous SMA iPSCs will require their genetic correction, thus we investigated also a novel strategy to ex-vivo genetically correct the cells. We generated iPSCs from fibroblasts from patients with SMA as well as from heterozygous carriers and wild-type healthy subjects using a non-viral method. Cells were nucleofected with oriP/EBNA1 vectors encoding six reprogramming factors. We used SMN2 sequence-specific ODNs to direct the exchange of a T to C at position +6 of exon 7, thus converting SMN2 into a SMN1-like gene in the SMA-iPSCs. SMA corrected cell lines contained no exogenous sequences and were indistinguishable from healthy iPSCs. The iPSCs were characterized and differentiated using a protocol to induce motoneuron commitment. The phenotype of cells was investigated by morphological, gene expression, and protein analysis. iPSC-purified motoneurons (SMA, WT and corrected SMA cells) were transplanted into the spinal cords of SMA mice. Non-viral SMA-iPSC-derived motor neurons reproduced disease-specific features (reductions in cell number, cell size, and axon length) while corrected SMA-specific-iPSCs gave rise to phenotypically rescued motor neurons in vitro and in vivo after transplantation in SMA spinal cord. Different splicing profiles, determined in vitro by microarray analysis in SMA motor neurons compared to wild-type, were normalized after the genetic correction. Transplantation of wild-type and corrected SMA motor neurons extended lifespan (> 50%) and ameliorated the phenotype of SMA mice significantly more than SMA motor neurons and untreated animals. These results offer a proof of concept that generating patient-specific corrected iPSCs and motor neurons free of exogenous elements is possible, as well as their therapeutic potential benefit for SMA.

ABSTRACT N. 96

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SETTE CLAUDIO**	
Telethon grant N.	**GGP09154**	
Total budget €	**198.100**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

REGULATION OF THE ALTERNATIVE SPLICING OF SMN2 BY SAM68 AND ITS IMPLICATION IN SPINAL MUSCULAR ATROPHY

Pagliarini Vittoria (2), Bustamante Maria Blaire (1), Compagnucci Claudia (1,2), Sette Claudio (1,2)

(1) Dipartimento di Biomedicina e Prevenzione, Università di Roma Tor Vergata - Via Montpellier, 1, 00133 Roma Tel. 06-72596260 Fax 06-72596268
(2) Laboratorio di Neuroembriologia, IRCSS Fondazione Santa Lucia, Roma

Spinal Muscular Atrophy (SMA) represents the primary genetic cause of infant mortality. This neuromuscular disorder is determined by loss of function mutations in the SMN1 gene, which cause deficiency in the Survival Motor Neuron (SMN) protein and degeneration of alpha-motor neurons in the spinal cord. SMN2, a nearly identical gene present in humans, mostly expresses a truncated and unstable SMN protein, due to skipping of exon 7 during the processing of the pre-mRNA. Nevertheless, the possibility of rescuing exon 7 splicing in SMN2 offers a therapeutic opportunity to restore the expression of full length SMN protein to ameliorate the disease symptoms. We have previously identified the RNA-binding protein Sam68 as a crucial regulator of SMN2 exon7 skipping and demonstrated that inhibition of Sam68 activity rescued exon 7 inclusion and SMN protein expression in SMA fibroblasts. We are currently testing the role of Sam68 in a neuronal context, which is more relevant for the SMA pathology. In particular, given their potential therapeutic value in regenerative medicine, we have performed our studies in Neural Stem Cells (NSCs). Isolation of NSCs from Sam68 knockout mice showed that ablation of this gene does not impair their ability to generate neurons, suggesting that interfering with the function of this splicing factor is not detrimental for neurogenesis. Interestingly, disrupting Sam68 activity even led to increased differentiation and augmented complexity of the neurons. These effects were correlated with a slight decrease in the size of the proliferating neurospheres and with no changes in the survival rate of wild type and knockout cells. NSCs were also established from wild type and type I SMA mouse embryos and analyzed for SMN2 splicing following infection with a Sam68 dominant-negative mutant protein. Expression of the Sam68 mutant protein promoted splicing of exon 7 in the SMN2 mRNA, suggesting that impairment of Sam68 function can rescue SMN expression also in a neuronal context. As an additional approach to correct SMN2 splicing, we have employed HDAC inhibitors, which were previously shown to partially rescue SMN expression in mouse models of type I SMA. Since epigenetic modification of the chromatin can sensibly influence splicing regulation, we tested several HDAC inhibitors for their ability to modulate SMN2 splicing. We found that LBH589 was the strongest inducer of exon7 inclusion in both human SMA fibroblasts and murine SMA NSCs. Importantly, up-regulation of Sam68 completely suppressed the effect of LBH589 on SMN2 splicing, whereas expression of Sam68 dominant-negative mutant enhanced it. These finding support the existence of a correlation between histone acetylation and Sam68 function and suggest that inhibition of Sam68 function in SMA cells might reinforce therapeutic approaches to recover SMN2 exon7 splicing and SMN protein expression.

ABSTRACT N. 97

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**TIZIANO FRANCESCO DANILO**	
Telethon grant N.	**GGP12116**	
Total budget €	**313.400**	
Centres: **2**	*Duration (yrs):* **2**	*Starting year:* **2012**

MUSCULAR MIRNOME AND TRANSCRIPTOME ANALYSIS AS A TOOL FOR THE IDENTIFICATION OF BIOMARKERS IN SPINAL MUSCULAR ATROPHY

Di Marcotullio Lucia (2), Bertini Enrico (3), Morandi Lucia (4), Baranello Giovanni (5), Mercuri Eugenio (6), Messina Sonia (7), Mora Marina (4), Di Pietro Lorena (1), Infante Paola (2), Moroni Isabella (5), D'Amico Davide (2), Gulino Alberto (2), D'Amico Adele (3), Abiusi Emanuela (1), Fiori Stefania (1), Neri Giovanni (1), Tiziano Francesco Danilo (1)

(1) Istituto di Genetica Medica, Università Cattolica, Roma - Largo Francesco Vito, 1 00168 Roma, Tel. 0630154606 Fax 0630157223; E-mail: fdtiziano@rm.unicatt.it
(2) Dipartimento di Medicina Molecolare, Università Sapienza, Roma
(3) Unità malattie neuromuscolari e neurodegenerative, Ospedale Bambin Gesù, Roma
(4) Unità malattie neuromuscolari, Istituto C. Besta, Milano
(5) Unità Neurologia dello Sviluppo, Istituto C. Besta, Milano
(6) Unità di Neuropsichiatria Infantile, Università Cattolica, Roma
(7) Dipartimento Neuroscienze, Università di Messina, Messina

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular condition, caused by SMN1 gene mutations (Lefebvre S.

et al., 1995) and characterized by muscle atrophy due to spinal alpha-motor neuron degeneration. However, several experimental lines of evidence support a pathogenic role of skeletal muscle in the condition (Guettier-Sigrist et al., 2001; Cifuentes-Diaz et al., 2001; Walker et al., 2008; Martinez-Hernandez et al, 2009; Mutsaers et al., 2011).
At present, although no cure for SMA is available, several therapeutic approaches are in the pipeline: outcome measures suitable for the evaluation of patients enrolled in clinical trials have been developed and validated, but the identification of reliable biomarkers is still elusive.
The main aim of the present project is to identify and validate RNA-based biomarkers for SMA, independent of SMN levels. To this aim, we will compare miRNome and transcriptome of muscle cells/biopsies of patients and controls, in order to identify differentially expressed RNAs. Those transcripts will be quantified in serum (miRNAs) and blood (mRNAs) samples from 60 patients affected from SMA I-III, and a similar number of age-matched controls. RNA levels will be related with the motor performance of patients, as evaluated by validated outcome measures.
The miRNome and transcriptome of myoblast and myotube cultures, as well as of muscle biopsies, from patients and controls will be determined by next generation sequencing and validated by real time PCR. The levels of differentially expressed RNAs will be determined in samples from patients, whose motor performance will be evaluated with different outcome measures, selected on the basis of age and severity of patients. So far, we have performed miRNome analysis of 10 myoblast cultures (5 patients and 5 controls) and the validation by real-time PCR is ongoing. About 30 miRNAs have been found differentially regulated in cultures from patients and controls. Transcriptome analysis is still ongoing.
Beside the strictly molecular part, this project will include also the clinical evaluations of SMA patients that will be performed thanks to the collaboration of some of the most relevant Italian neuromuscular centers, involved in the follow-up of SMA patients.
This project has strong potential translational implications, since may quickly lead to the identification and validation of prognostic and pharmacodynamic biomarkers for SMA, suitable for both clinical and pre-clinical studies.

ABSTRACT N. 98

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**VITA GIUSEPPE**	
Telethon grant N.	**GUP10008**	
Total budget €	**170.300**	
Centres: **8**	*Duration (yrs):* **2**	*Starting year:* **2011**

NOVEL OUTCOME MEASURES FOR CHARCOT-MARIE-TOOTH DISEASE

Vita Giuseppe (1), Fabrizi Gian Maria (2), Gemignani Franco (3), Padua Luca (4), Pareyson Davide (5), Quattrone Aldo (6), Santoro Lucio (7), Schenone Angelo (8), Mazzeo Anna (1), Russo Massimo (1), Stancanelli Claudia (1), Gentile Luca (1), Cavallaro Tiziana (2), Vitetta Francesca (3), Contini Mara (3), Pazzaglia Costanza (4), Russo Giuseppina (4), Iacovelli Chiara (4), Piscosquito Giuseppe (5), Calabrese Daniela (5), Valentino Paola (6), Manganelli Fiore (7), Pisciotta Chiara (7), Monti Bragadin Margherita (8), Aiello Alessia (8), Bolla Simone (8)

(1) Dipartimento di Neuroscienze, Università di Messina, Tel. 090.2212793; Fax 090.2212789; E-mail: vitag@unime.it
(2) Dipartimento di Scienze Neurologiche, Neuropsicologiche, Morfologiche e Motorie, Università di Verona
(3) Dipartimento di Neuroscienze, Università di Parma
(4) Fondazione Don Carlo Gnocchi ONLUS, Milano
(5) Fondazione I.R.C.C.S., Istituto Neurologico "Carlo Besta", Milano
(6) Dipartimento di Scienze Mediche, Università "Magna Graecia", Catanzaro
(7) Dipartimento di Scienze Neurologiche, Università "Federico II", Napoli
(8) Dipartimento di Neuroscienze, Oftalmologia e Genetica, Università di Genova

Objective: To validate the 6-minute walk test (6MWT) and the StepWatchTM Activity Monitor (SAM), two tools reflecting patients' activities of daily living, in combination with other already validated outcome measures in a wide and well-represented sample of Charcot-Marie-Tooth (CMT) patients.
Background/Rationale: CMT is the most common inherited neuromuscular disorder. Few clinical trials and natural history studies have been performed so far in CMT1A. Some outcome measures have been recently identified and validated. Two tools that most likely reflect accurately patient motor performances in daily living, but not yet validated in CMT patients, are the 6MWT and the SAM. They have been widely employed to assess functional exercise capacity in neurological diseases and in some other neuromuscular disorders.
Project description: We have proposed a prospective follow-up (12 months) of 200 CMT patients (CMT1A, CMT1B, X-linked CMT) with multiple outcome measures. The project is articulated in the following steps: 1) training of the physicians from each of the participating centers and assessment of the inter- and intra-rater reliability of 6MWT and SAM; 2) clinical and electrophysiological assessments, with 6MWT, SAM, CMTNS, 10-meter timed walking test, distal arm and leg strength measured by MVIC and SF-36 health related quality of life scale at baseline and after 6 and 12 months; 3) Interim analysis at 6 months; 4) Final data analysis. Considering the slow disease progression, if final data analysis will show no clear trend in the results of the selected outcome measures, the network will propose a continuation of the study up to 24 months of follow-up even without further financial support.
Study update: training of the physicians from each of the participating centers has been completed in January 2012. In April 2012 first patients have been enrolled and they are going to have the six month evaluation in the next few days. Patients' compliance and collaboration to the study have been quite good. The SAM application requires no more than 2-3 minutes, and same time is needed to collect the data after the five days of registration. 6MWT needs little effort of the patients, and obviously about ten minutes to be performed. All the clinical and electrophysiological assessments are performed in about one hour.
So far 80 CMT patients have been enrolled: 55 of them have CMT1A, 12 CMT1B, 13 CMTX.
Anticipated output: such study will allow to validate two new outcome measures available for forthcoming clinical trials and to select tools adequately powered to detect significant changes in a disease with a slowly progressive course.
Collaborations: Prof. M. Reilly (University College of London, UK) will contribute to the project, as external collaborator, in performing comparisons between SAM and SenseWear Pro 3 Armband outputs. ACMT-RETE, a web-based non-profit volunteer patients' association, will provide any useful support in favouring recruitment and partecipation to the study and in the dissemination of related informations among the association's members.

ABSTRACT N. 99

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FERRARIN MAURIZIO**	
Telethon grant N.	**GUP10010**	
Total budget €	**283.775**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2011**

DEVELOPMENT OF AN INSTRUMENTED MOVEMENT ANALYSIS PROTOCOL FOR THE MULTITASKING ANALYSIS OF LOCOMOTOR FUNCTIONS IN ADULT AND YOUNG PATIENTS WITH CHARCOT-MARIE-TOOTH DISEASE: MULTICENTER STUDY TO CHARACTERISE RELIABILITY AND RESPONSIVENESS

Lencioni Tiziana (1), Beghi Ettore (7), Di Sipio Enrica (4), Forni Marco (5), Minciotti Ileana (4), Moroni Isabella (3), Padua Luca (4), Pagliano Emanuela (3), Pareyson Davide (2), Pazzaglia Costanza (4), Piscosquito Giuseppe (2), Rabuffetti Marco (1), Russo Giuseppina (4), Schenone Angelo (6), Ferrarin Maurizio (1)

(1) Polo Tecnologico, IRCCS Fondazione Don Gnocchi Onlus, Via Capecelatro 66, 20148 Milano, Tel. +39-02-40308305, Fax +39-02-4048919, E-mail: mferrarin@dongnocchi.it
(2) Clinica delle neuropatie degenerative centrali e periferiche, IRCCS Istituto Neurologico Besta, Milano
(3) Dipartimento di Neuroscienze Pediatriche, IRCCS Istituto Neurologico Besta, Milano
(4) Centro S. Maria della Pace, Fondazione Don Gnocchi Onlus, Roma
(5) Polo Riabilitativo del Levante Ligure, Fondazione Don Gnocchi Onlus, Roma
(6) Dipartimento di Neuroscienze, Oftalmologia e Genetica, Università di Genova, Genova
(7) Laboratorio Malattie Neurologiche, Istituto di Ricerche Farmacologiche Mario Negri, Milano

Introduction: Charcot-Marie-Tooth (CMT) disease is an inherited neuropathy affecting the peripheral nervous system, characterized by a slowly progressive course. Usually onset of symptoms occurs in youth before the age of 20 years. Currently CMT natural history has not yet been well characterized. Goal of the project is to study, through instrumented Movement Analysis (MA), a group of CMT pa-

tients, to analyse the correlation of MA parameters with demographic, genetic and clinical characteristics and to evaluate MA as an outcome measure for CMT studies.
Methods: 100 CMT subjects have been recruited in three different sites, Milan, Rome and Sarzana, involved in this study and have been divided into two groups according to age: young (aged 6-17 years) and adults (aged 18-70years). The sample is genetically characterized as follows: 55 CMT1A adults, 15 CMTX1 adults and 30 CMT1A young. Currently 40 healthy controls have also been recruited, 21 adults and 19 young. Each patient underwent a clinical examination and a biomechanical evaluation using MA including the following locomotor tasks: natural and increased speed walking, toe- and heel-walking, stair ascending and descending, and postural stabilization after the sit-to-stand transition. In a subgroup of 20 CMT subjects, a 4-6 weeks test-retest reliability study was also performed on MA data.
Results: The test-retest reliability of MA, showed good repeatability in most of CMT-specific parameters with absolute angular errors of less than 5°, that represents a clinically acceptable threshold.
We have identified two biomechanical parameters able to quantify the two primary deficits related to CMT: foot-drop and plantar flexor failure. In both young and adult groups, cluster analysis allowed us to evidence three subgroups of CMT patients which showed specific locomotor behaviours during natural walking: 1) normal-like patients (NL), not significantly different from controls in any parameter; 2) foot-drop patients (FD), where the only significant alteration was the deficit of ankle dorsiflexion during swing; 3) patients with foot-drop and push-off failure (FD&POD), where a significant reduction of plantarflexion power at push-off was evidenced in addition to foot-drop. More challenging tasks, such as toe-heel walking, showed deficits related to CMT also in adult and young NL patients ($p<0.05$ from controls). The genotype of CMT patients in the adult heterogeneous group was not crucial for the characterization of their locomotor pattern.
Discussion: The MA can be used as a functional outcome measure in CMT. The analysis of the primary signs leads to the following hypothesis: symptoms are associated with disease progression, such as a transition from the mild stage, NL, to the more severe, FD&POD, through the intermediate FD. This hypothesis will be verified at 12 and 24 months follow-up evaluations. Furthermore, the functional classification identified may be important for a correct therapeutic approach.

ABSTRACT N. 100

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SCHENONE ANGELO**	
Telethon grant N.	**GUP09013**	
Total budget €	**124.500**	
Centres: **4**	*Duration (yrs):* **2**	*Starting year:* **2010**

A MULTICENTER STUDY TO EVALUATE THE EFFECTS ON CHARCOT-MARIE-TOOTH NEUROPATHY TYPE 1A OF A COMPOSITE TREADMILL, STRETCHING AND PROPRIOCEPTIVE EXERCISE (TRESPE) REHABILITATION PROGRAM

Schenone Angelo (1), Monti Bragadin Margherita (1), Pareyson Davide (2), Padua Luca (3, 4), Fabrizi Gianmaria (5)

(1) Department of Neuroscience, Ophthalmology, Rehabilitation, Genetics and Mother and Child Sciences University of Genoa, Largo Daneo 3, Genova, Italy, Tel. 010.3537040, Fax 010.3538639, E-mail: aschenone@neurologia.unige.it
(2) Clinic of Central and Peripheral Degenerative Neuropathies, Dept of Clinical Neurosciences, IRCCS Foundation, C. Besta Neurological Institute, Milan
(3) Department of Neuroscience, Institute of Neurology, Catholic University, Rome, Italy
(4) Centro S. Maria della Pace, Don Carlo Gnocchi Onlus Foundation, Italy
(5) Department of Neurological and Visual Sciences, University of Verona, Italy

The safety and efficacy of rehabilitation in Charcot-Marie-Tooth (CMT) hereditary neuropathy is unclear. In particular, there is no information on the use of aerobic exercising in CMT1A.
Aims: 1) clarify if aerobic exercise, based on a tightly controlled program at the treadmill, is well tolerated by CMT1A patients and, eventually, may improve their ability to walk; 2) understand whether the respiratory function and cardiopulmonary response to exercise is affected in CMT1A; 3) study the maintenance of improvement, if any, in a six months follow up (FU) time; 4) evaluate the impact of rehabilitation on the quality of life in these patients.
The TreSPE study is a multicentre, prospective, randomised, single blind, controlled rehabilitation trial in CMT1A patients.
Outcome measures are: 1) the 10m timed walking test (primary outcome measure), as aerobic exercise previously showed some effect on walking ability of CMT patients. 2) 12-item walking scale, 3) distal strength determined with a myometer; 4) SF36 health-related QoL, 5) ankle angle measured by a gonyometer, 6) Berg Balance and Mobility scale, 7) maximal expiratory pressure, forced vital capacity and maximal inspiratory capacity; peak oxygen consumption during incremental exercise tests on treadmill (secondary outcome measures). Daily physical activity and fatigue will be also evaluated.
Expected output: 1) validate the use of treadmill in CMT1A; 2) standardize rehabilitation protocols and outcome measures for CMT1A, to be used as gold standard in future studies; 3) avoid expensive and time-consuming therapies in CMT1A, in case of negative results.
To date we have enrolled 57 patients. All of them underwent the complete assessment at T1 and started the treatment being divided the in two groups: 1) Treadmill, Stretching and Proprioceptive exercises (TreSPE); 2) Stretching and Proprioceptive exercises (SPE). As the enrollment is still ongoing and the study blind, we will present the baseline data regarding 57 patients.

ABSTRACT N. 101

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BRUZZONE SANTINA**	
Telethon grant N.	**GGP12002**	
Total budget €	**115.500**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

ROLE OF PURINERGIC RECEPTORS IN MYELINATION: THERAPEUTIC IMPLICATIONS FOR TREATMENT OF THE PERIPHERAL NEUROPATHY CHARCOT-MARIE-TOOTH 1A

Nobbio Lucilla (1), Visigalli Davide (1), Mannino Elena (1), Fiorese Fulvia (1), Kassack Matthias (2), Sturla Laura (1), Pareyson Davide (3), Sereda Michael W (4), Prada Valeria (1), Mancardi Gianluigi (1), Zocchi Elena (1), Schenone Angelo (1), Bruzzone Santina (1)

(1) Dept of Experimental Medicine, Section of Biochemistry and CEBR, Viale Benedetto XV, 7 16132, Genova, Italy, Tel. +39.010.3538161; Fax +39.010.353415; E-mail: santina.bruzzone@unige.it
(2) Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University of Düsseldorf, Germany
(3) Clinic of Central and Peripheral Degenerative Neuropathies Unit, C. Besta Neurological Institute, Milano
(4) Max-Planck-Institute of Experimental Medicine, Göttingen, Germany

Charcot-Marie-Tooth 1A (CMT1A) is a demyelinating hereditary neuropathy whose pathomechanisms are still poorly defined. As a consequence, etiologic treatments are not yet available for CMT1A. We reported an abnormally high intracellular $Ca2+$ concentration in Schwann cells (SC) from a rat model of CMT1A (CMT1A SC), caused by overexpression of the purinergic receptor P2X7. Correction of the elevated $Ca2+$ levels through down-regulation of P2X7 restored the normal phenotype in cultured CMT1A SC.
In this study, organotypic dorsal root ganglia (DRG) cultures from both wild type and CMT1A rats were treated with A438079, a commercial P2X7 antagonist, which significantly ameliorated myelination in CMT1A cultures, as evaluated by expression levels of the myelin protein MPZ and by morphometric analysis of myelin segment density.
An Ap2A (diadenosine diphosphate) isomer (P18), which behaves both as an antagonist of P2X7 and as an agonist of P2Y11 [Bruzzone S et al, J Biol Chem 285: 21165-74, 2010], was capable of increasing the intracellular cAMP content ([cAMP]i) in CMT1A SC by activating P2Y11 and also improved myelination in DRG cultures. Moreover, we found that in CMT1A SC the basal [cAMP]i is significantly lower than in wild type cells, in line with the established role for cAMP in regulating SC differentiation and myelination. Interestingly, P18 also decreased the amount of nonphosphorylated neurofilaments, used as a marker of axonal sufferance.
These results suggest that P2X7 antagonists could represent a therapeutic strategy aimed at correcting the molecular derangements causing demyelination in CMT1A. To reinforce this hypothesis we also showed that P2X7 is present in normal human SC and axons, and overexpressed in nerve tissue from CMT1A rats and patients.

ABSTRACT N. 102

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BUCCI CECILIA**	
Telethon grant N.	**GGP09045**	
Total budget €	**165.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

MOLECULAR BASIS OF CHARCOT MARIE TOOTH TYPE 2B DISEASE

Cogli Laura, Bramato Roberta, Donno Claudia, Margiotta Azzurra, Paiano Aurora, Bucci Cecilia

Dip. Scienze e Tecnologie Biologiche ed Ambientali (DiSTeBA), Università del Salento, Via provinciale Monteroni 165, 73100 Lecce, Tel. 0832.298900, Fax 0832.298626; E-mail: cecilia.bucci@unisalento.it

Charcot-Marie-Tooth disease (CMT) is the most common group of inherited peripheral neuropathies. More than 40 genetic loci associated with different forms of the disorders have been identified. CMT type 2B (CMT2B) is an axonal non-demyelinating form characterized by distal muscle weakness and atrophy, mild sensory loss, normal or near-normal nerve conduction velocity, and frequent ulcerations that often lead to amputations. Four missense mutations that target highly conserved amino acids in Rab7 cause the Charcot-Marie-Tooth 2B disease. Rab7 is a small GTPase, evolutionarily conserved from yeast to human and expressed in all tissues, controlling transport to endocytic degradative compartments,
We have previously demonstrated that CMT2B-causative Rab7 mutant proteins have higher Koff for nucleotides compared to the wt protein (particularly high for GDP) and, as a consequence, lower GTPase activity. These mutant proteins are predominantly GTP-bound in the cells, and are able to rescue Rab7 function when expressed in Rab7-silenced cells.
However, it is still unclear how mutations in a ubiquitous protein affect specifically peripheral neurons. A possibility is that Rab7 controls specific pathways present in peripheral neurons or that CMT2B-causing Rab7 mutations alter the interaction of Rab7 with specific effectors present only in these cells. Interestingly, we found that expression of CMT2B-causing Rab7 mutants in PC12 and in Neuro2A cells impairs neurite outgrowth and neuronal differentiation, measured as up-regulation of GAP43 in PC12 cells and of NeuN in Neuro2A cells. Also, using the two-hybrid system, we identified, in a dorsal root ganglia (DRG) cDNA library, peripherin as a Rab7 effector. Peripherin is a type III intermediate filament protein expressed predominantly in the peripheral nervous system. In injured nerves a peripherin increase has been observed, suggesting that peripherin plays a role in axonal regeneration and nerve repair. In particular, peripherin may be involved in the regrowth of axons. The interaction with Rab7 was confirmed by co-immunoprecipitation and pull-down assays, and we demonstrated that this interaction is direct using bacterially expressed recombinant proteins. Silencing or overexpression of Rab7 changed the soluble/insoluble rate of peripherin indicating that Rab7 is important for peripherin organization and assembly. In addition, disease-causing Rab7 mutant proteins bind more strongly to peripherin and cause a significant increase in the amount of soluble peripherin. As peripherin seems to play a role in axonal regeneration after injury, these data suggest that altered interaction between Rab7 and peripherin could affect axonal regeneration. Also, as peripherin coassembles with neurofilaments (that are mutated in other forms of CMT), alterations of peripherin organization caused by Rab7 mutants could affect neurofilaments organization and functions, which are fundamental for motor and sensory neurons.

ABSTRACT N. 103

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**FELTRI MARIA LAURA**	
Telethon grant N.	**GGP08021**	
Total budget €	**376.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

LAMININS AND THEIR RECEPTORS IN HEREDITARY NEUROPATHIES

Colombelli Cristina (1), Pavoni Ernesto (1), Zambroni Desiree (1), Palmisano Marilena (2), Occhi Simona (1), Nicole Sophie (3), Soininen Raija (4), Eshed Yael (5), Peles Elior (5), Wrabetz Lawrence (1,2), Feltri Maria Laura (1,2)

(1) Division of Genetics and Cell Biology, Via Olgettina 58, San Raffaele Scientific Institute, 20132 Milano, Italy, Tel. 02.26434782, Fax 02.26434767, E-mail:feltri.laura@hsr.it
(2) 5 Hunter James Kelly Research Institute, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, NY, USA
(3) Inserm, U546, Univ Paris 06, Paris, France
(4) Oulu Center for Cell-Matrix Research, Biocenter Oulu, Department of Medical Biochemistry and Molecular Biology, University of Oulu, Finland
(5) Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

The dystroglycan complex links extracellular laminins to the cytoskeleton in different cells. Components of the complex in muscle cells are the target of multiple muscular dystrophies. Similarly we have shown during the course of the 3 years that the abnormalities described in diverse hereditary neuropathies (Charcot-Marie-Tooth 4F due to periaxin deficiency, congenital muscular dystrophies with peripheral nervous system involvement: MDC-1A, Fukuyama/MDC1C and MDC1D due to laminin-2, fukutin/FKRP or LARGE glycosyltransferase deficiencies) share a common pathogenesis based on misfunction of the dystroglycan complex.
Here we show that proteolysis of dystroglycan by furin is required for proper clusterization of sodium channels at Nodes of Ranvier. Saltatory conduction requires clustering of voltage-gated sodium channel at nodes of Ranvier. We previously showed that specific laminin isoforms and dystroglycan (DG) complexes are located around peripheral node and are required for proper microvilli formation and sodium channel clustering in humans and mice. However the mechanims by which dystroglycan aids sodium channel clustering is unknown. Here we show that the DG complex localizes as early as ERM proteins at nascent nodes, it is dispensable for initial clustering of sodium channel, but it is required for proper compaction of nascent sodium channel clusters at heminodes and nodes. By immunoelectron microscopy the extracellular a-DG is found in Schwann cell microvilli adjoining both the basal lamina and the axon (nodal gap). The N-terminal domain of a-DG is cleaved by furin and secreted by Schwann cells, and colocalize with glial NrCAM and gliomedin clusters when Schwann cells align on axons and begin to myelinate. We find that the nodal gap also contains heparin-sulfate proteoglycans that bind dystroglycan, agrin and perlecan; in addition to syndecan 3 and versican1. Deletion of dystroglycan in Schwann cells reduces versican 1 and perlecan at node, but genetic alteration of perlecan alone (hypomorphic mutations or ablation of the heparin-sulfate attachment site) are not sufficient to alter sodium channel clustering in mice. Thus we propose that full-length and furin-shed dystroglycan participate in clusterization of sodium channel at heminodes and nodes by multiple mechanisms: full length DG binds laminins in the basal lamina and contributes to microvilli organization, while the a-DG N-terminal fragment is cleaved by furin and connects NrCam and gliomedin to proteoglycans in the nodal gap matrix. These data also show that extracellular matrix molecules are important factors in the clusterization of sodium channels at nodes of Ranvier.

ABSTRACT N. 104

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**TAVEGGIA CARLA**	
Telethon grant N.	**GPP10007**	
Total budget €	**1.442.850**	
Centres: **5**	*Duration (yrs):* **3**	*Starting year:* **2010**

MODULATING NEUREGULIN-1 SIGNALS TO TREAT HEREDITARY DEMYELINATING NEUROPATHIES

Feltri Maria Laura (2), Pavoni Ernesto (2), Previtali Stefano C. (1), Wrabetz Lawrence (2), D'Antonio Maurizio (2), Bolino Alessandra (1,3), Taveggia Carla (1)

(1) Division of Neuroscience San Raffaele Scientific Institute and INSPE San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy, Tel. +39.02.26434439, Fax +39.02.26436164, E-mail: taveggia.carla@hsr.it
(2) Division of Genetics and Cell Biology San Raffaele Scientific Institute, Milano, Italy
(3) Dulbecco Telethon Institute

CMT neuropathies are the most frequent human hereditary neuropathies and are associated with mutations in more than 30 genes. Modes of transmission include: recessive, dominant, or X-linked, with recessive diseases being more severe and of earlier onset. The severe disability and motor impairment derive mainly from progressive axonal damage and loss, with inefficient regeneration. There is

no adequate treatment for CMT neuropathies and so far only a handful of clinical trials have been undertaken. Many CMT neuropathies display altered levels of myelination. Therefore therapeutical strategies aiming at restoring normal myelin levels could produce an effective approach for their collective treatment. Furthermore, as myelin is neuroprotective, restoring its normal levels could facilitate the maintenance of a functional Schwann cell-axon unit and reduce axonal damage, which is the ultimate cause of morbidity.
Studies from the Coordinator and other groups have shown that NRG1 type III is an essential instructive signal for peripheral myelination. NRG1 type III drives the binary choice between myelination and non-myelination by Schwann cells. In addition, the amount of axonal NRG1 type III determines the thickness of the myelin sheath. Thus, modulating axonal NRG1 levels in both hypo- and hyper-myelinating hereditary neuropathies could be a feasible approach to normalize myelination in several CMT neuropathies with diverse pathogenesis.
Previous studies have shown that NRG1 type III activity is regulated by the cleavage of secretases. In particular the ß-secretase BACE1 activates NRG1 type III enhancing myelination. The Coordinator has recently published that NRG1 type III is also cleaved by the pharmacologically accessible a-secretase TACE to inhibit myelination.
In these studies we proposed to explore the mechanisms of action of NRG1 type III and TACE in the array of preclinical animal models that we have generated and characterized over the years, among them: models of CMT1B neuropathies, showing congenital or later-onset hypomyelination (Partner 3) and of CMT4B neuropathies, showing redundant hypermyelination (Partner 4). We will also investigate whether NRG1 type III cooperates with components of the extracellular matrix and of the cytoskeleton (Partner 1 and 2).
Our results indicate that in vitro and in vivo modulation of NRG1 type III levels is effective in modulating myelination in hyper- and hypo-myelinating neuropathies. We also have evidences suggesting that the extracellular matrix and the cytoskeleton modulate and integrate signals from NRG1 to regulate myelination. Further, the use of TACE inhibitors or activators can effectively control myelination in vitro in a Schwann cell neuronal coculture system. Preclinical trials are ongoing to validate our results in preclinical animal models of hypo and hyper-myelinating neuropathies.

ABSTRACT N. 105

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BORTOLOZZI MARIO**	
Telethon grant N.	**GGP12269**	
Total budget €	**341.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2013**

STRUCTURAL AND FUNCTIONAL ANALYSIS OF SELECTED CONNEXIN32 MUTATIONS IMPLICATED IN THE PATHOGENESIS OF THE X-LINKED FORM OF CHARCOT-MARIE-TOOTH DISEASE

Crispino Giulia (1), Mammano Fabio (1,2), Bortolozzi Mario (1,2)

(1) Istituto Veneto di Medicina Molecolare (Vimm), Via G.Orus 2, 35129, Padova, Italy, Tel. 049.7923247, Fax 049.7923250, E-mail: mario.bortolozzi@gmail.com
(2) Dipartimento di Fisica e Astronomia "G.Galilei", Università di Padova

Charcot-Marie-Tooth (CMT) disease is the most common genetic disorder of the peripheral nervous system. This devastating condition, for which there is no cure, affects about 1/3000 individuals, of which 10-12% carry the X-linked form, called CMT1X disease. Over 400 different genetic mutations associated with CMT1X disease have been identified in chromosome X, and in particular in the gene that encodes connexin32 protein (Cx32). This is a tetraspan membrane protein which forms reflexive junctional channels in the Schwann cell myelin sheath, providing a radial and fast pathway for ions and signalling molecules which are critical to maintain correct myelination. Despite the availability of a huge number of studies on normal and mutated Cx32 expression and function, a molecular interpretative framework of the observed CMT1X phenotype is still lacking but we believe that a common explanation of the disease could be provided by the identification of a limited number of cytoplasmic molecules crucial for Schwann cell myelination and having impaired trafficking through Cx32 mutant channels. Oh et al.(Neuron, 1997) proposed cAMP as the best candidate molecule having impaired trafficking as elevations of cAMP in Schwann cells, which may result from direct axonal–Schwann cell contact, are at least partially involved in the induction of genes necessary for myelination (Wang et. al, Neurobiol Dis, 2004).
We have thus performed experiments in HeLa cells by a combination of double patch-clamp and FRET imaging of the cAMP sensor H30 to determine whether an electrically functional mutant channel of human Cx32 (R220stop) could indeed present with a reduced permeability to the second messenger cAMP. The measured single channel conductance of Cx32R220stop mutant resulted close to that of the wild type (WT) channel, meaning that K+ permeability is not impaired by the mutation. By contrast, we found a significantly reduced permeability to cAMP in the mutant compared to the WT channel. Similar experiments with the exogenous fluorescent tracers lucifer yellow (LY) and calcein show that the Cx32R220stop mutant is far less permeable to LY and calcein than the WT channel, in accord with the (first time in literature) observed reduced permeability to cAMP and also in agreement with prior measurement using LY and calcein (Bicego et al., Neurobiol Dis, 2006).
This knowledge will provide crucial insight for the future course of this Telethon project, in particular in the study of Cx32 mutations in a new cellular system based on cultured Schwann cells derived from stem cells of CMT1X patients.

ABSTRACT N. 106

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CATTANEO ANTONINO**	
Telethon grant N.	**GGP11179**	
Total budget €	**189.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

TOWARDS AN NGF-BASED THERAPY FOR HEREDITARY SENSORY AND AUTONOMIC NEUROPATHIES IV AND V

Pancrazi Laura (1), Capsoni Simona (1), Costa Mario (2), Cattaneo Antonino (1)

(1) Laboratory of Neurobiology, Scuola Normale Superiore, Piazza dei Cavalieri 7, 56126 Pisa, Italy Tel. 050.309320, Fax 050.563513, E-mail: antonino.cattaneo@sns.it
(2) Institute of Neuroscience, National Research Council (CNR), Pisa

The Hereditary Sensory and Autonomic Neuropathies (HSAN) IV and V are rare life-threatening autosomal recessive disorders characterized by a complete lack of pain sensation and are linked to defects in Nerve Growth Factor (NGF) signalling. HSAN IV is clinically characterized by absence of sweating and total lack of reactions to painful stimuli leading to self-mutilation, burn injuries, multiple fractures, and neuropathic joints. It is caused by a rather wide spectrum of mutations in the TrkA gene, encoding for the tyrosine kinase receptor of NGF.
Patients affected by HSAN V have a selective congenital loss of pain and temperature sensation leading to painless fractures, bone necrosis, osteochondritis, and neuropathic joint destructions. Sweating is normal. The responsible genetic mutation is the R100W point mutation in the NGF gene. It is noteworthy that while HSAN IV patients suffer from severe mental retardation, HSAN V patients are cognitively normal.
The aim of our project is to study the mechanisms at the basis of the differentially altered NGF signalling in these two diseases.
To reach this goal we are adopting two different strategies.
The first strategy consists in comparing the signal transduction pathway downstream TrkA wt and TrkA D668Y in cell cultures. Indeed, D668Y is the most common HSAN IV mutation and, differently from other mutants, NGF-induced TrkA phosphorylation in D668Y mutants has been reported to be comparable to that of wild type TrkA. However, it was unknown whether, as in the case of HSAN V, downstream signaling of TrkA is impaired. To answer this question we have generated two expression plasmids harbouring the cDNA of the human TrkA gene in its wild type form and carrying the D668Y substitution, respectively. Our preliminary data in transfected SHSY5Y suggest that downstream signalling in D668Y mutants is not affected, as measured by Plc gamma and Mapk phosphorylation after NGF treatment. TrkA phosphorylation levels induced by NGF treatment are confirmed to be comparable in wild type and D668Y mutant receptors.
The second approach consists in investigating the NGF signalling in HSAN IV and HSAN V animal models. To this aim we have generated a targeting vector in order to produce a line of knock-in mice in which the murine TrkA sequence is disrupted and replaced by the complete coding sequence of the human ortholog TrkA carrying the D668Y mutation. Positive ES recombinant clones have been selected and are being injected into host blastocysts.
Further, we are generating the targeting vectors for two knock-in mouse lines in which the NGF coding sequence is replaced by the

cDNA of the human NGF either in its wild type form or carrying the R100W mutation.

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ABSTRACT N. 107

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PINTON PAOLO**	
Telethon grant N.	**GGP09128**	
Total budget €	**263.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

INVOLVEMENT OF MITOCHONDRIAL PROTEINS IN AUTOPHAGY: A POSSIBLE LINK WITH MITOCHONDRIAL DISORDERS

Giorgi Carlotta (1), Baldassari Federica (1), Bononi Angela (1), Bonora Massimo (1), De Marchi Elena (1), Ioannidi Elli (1), Marchi Saverio (1), Missiroli Sonia (1), Morciano Giampaolo (1), Patergnani Simone (1), Poletti Federica (1), Rimessi Alessandro (1), Zanna Claudia (2), Rugolo Michela (3), Carelli Valerio (2), De Pinto Vito (4), Rosario Rizzuto (5), Pinton Paolo (1)

(1) Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Via L. Borsari 46, 44121 Ferrara Tel. 0532455802 E-mail: pnp@unife.it
(2) Department of Neurological Sciences, University of Bologna
(3) Department of Pharmacy and Biotechnology, University of Bologna
(4) Department of Chemical Sciences, University of Catania
(5) Department of Biomedical Sciences, University of Padua

In addition to the well-characterized energy-producing functions, mitochondria play a crucial role in promoting apoptosis and autophagy in response to specific signals. Mitochondrial and endoplasmic reticulum (ER) networks are fundamental for the maintenance of cellular homeostasis and for determination of cell fate under stress conditions. Zones of close contact between ER and mitochondria called MAM (mitochondria associated membranes) support communication between the two organelles including bioenergetics and cell survivor. In this project, we investigated:
i) The importance of MAM in the control of autophagy
ii) The involvement VDAC in autophagy
iii) The cross-talk between apoptosis and autophagy
iv) The role of the protein kinase C (PKC) in the control of the mitochondrial energy status and in the regulation of autophagy
v) The autophagy process in some models of mitochondrial disorders.

The data obtained underscore the importance of mitochondria in the regulation of autophagy. Moreover, the finding that a pharmacological modulation of the mitochondrial membrane potential modifies autophagy levels may be useful in fighting the pathological contexts that are characterized by deregulated levels of autophagy.

ABSTRACT N. 108

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**VERGANI LODOVICA**	
Telethon grant N.	**GGP10145**	
Total budget €	**161.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

ROLE OF MITOCHONDRIAL DYNAMIC AND AUTOPHAGY IN THE SEGREGATION OF MUTANT mtDNA

Sara Bettio, Adriana Malena, Lodovica Vergani

Department of Neurosciences SNPSRR, University of Padova, c/o Centro Biomedico Pietro D'Abano, Via Orus 2, 35100 Padova, Italy, Tel. +39 049 8216162; Fax +39 049 8216163, E-mail: lodovica.vergani@unipd.it

BACKGROUND The majority of human pathogenetic mtDNA mutations are heteroplasmic. In fact mutant mtDNA molecules frequently co-exist with wild-type copies. One common point mutation of mtDNA is A3243G, often associated with the neuromuscular disease MELAS. How deleterious mtDNA mutations become established, and how they wax and wane over the course of time are matters of intense investigation. Several reports have suggested that mutant mtDNAs become fixed purely by the stochastic process of genetic drift. However, recent studies in mouse suggest that a purification-selection process operates in the germline to weed out deleterious mtDNA variant. The surveillance system for the functional integrity of mtDNA may simply fail at low frequency enabling deleterious mutations to become established in rare cases, thereby producing pathological states; alternatively or additionally particular mtDNA variants, such as A3243G mtDNA, may evade the counter-selection measures acting against deleterious mutants. In somatic cells the distribution of mutant mtDNA has been shown to be non-random in many contexts. Several studies have shown that particular mtDNA variants undergo biased segregation in human cell cultures, in particular lung cells favour wild type mtDNA molecules, at difference of muscle cells that favour mutant mtDNA.

AIMS. The goal of the project is to clarify the relationship between mutant mtDNA segregation, autophagy and mitochondrial dynamic. To do that we performed studies on the:
1) Characterisation of autophagy: in lung and muscle cells harbouring A3243G mutant mtDNA, was evaluated the protein and expression level of BNIP3, BECLIN, LC3-II and p62.
2) Influence of mitochondrial dynamics on the segregation: in muscle heteroplasmic cells by downregulation of the fusion protein MFN1.

MATERIAL AND METHODS. 1) Characterisation of autophagy: in lung and muscle heteroplasmic cybrids with 0%-70%-80%-99% mutant A3243G mtDNA, and in the relative parental lines, the autophagic proteins BNIP3, BECLIN, LC3-II and p62 were analysed by RT-PCR for the expression level and by Western blotting (WB) for the protein amount, in cells treated with or w/o chloroquine, to measure the autophagic flux.
2) Influence of mitochondrial dynamics on the segregation: in muscle heteroplasmic cells, with 80% MELAS mutation, the fusion protein MFN1 was downregulated by RNAi technique. After selection, the effective reduction of RNA and protein of MFN1 was evaluated by RT-PCR and WB respectively. In the clones with a significant reduction of MFN1 compare to controls (cells transfected with empty vector), morphometric analysis of mitochondria and quantification of percentage of mutant mtDNA by hot-PCR were evaluated.

RESULTS. 1) Characterisation of autophagy: we found that the lung cells had an increased level of autophagic flux compare the muscle cells, suggesting a effective role of autophagy in the segregation of mtDNA. 2) Influence of mitochondrial dynamics on the segregation: 9 clones with downregulation of MFN1 and 9 control clones were selected. In all the cells with reduced MFN1, mitochondrial morphology indicated a significant increase of mitochondrial fission. In parallel in the same clones we didn't find a significant variation of percentage of mtDNA mutation, that was similar to controls. These data suggest that mitochondrial dynamic is necessary but not sufficient in the mtDNA segregation.

ABSTRACT N. 109

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**PALMIERI FERDINANDO**	
Telethon grant N.	**GGP11139**	
Total budget €	**395.900**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2011**

MITOCHONDRIAL ASPARTATE/GLUTAMATE CARRIER 1 DEFICIENCY: PATHOGENETIC MECHANISMS AND MUTATIONAL ANALYSIS

Palmieri Ferdinando (1), Lasorsa Francesco Massimo (2), Monti Barbara (3), Pinton Paolo (4), Zeviani Massimo (5)

(1) Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Tel 0805443323, Fax 0805442770, E-mail: ferdinando.palmieri@uniba.it
(2) Istituto di Biomembrane e Bioenergetica IBBE CNR, Bari
(3) Dipartimento di Farmacia e Biotecnologie, Università di Bologna
(4) Dipartimento di Morfologia, Chirurgia e Medicina Sperimentale, Università di Ferrara
(5) Fondazione IRCCS Istituto Neurologico "C. Besta", Milano

The mitochondrial aspartate/glutamate carrier (AGC) catalyzes a Ca^{2+}-stimulated unidirectional entry of glutamate into mitochondria in exchange for intramitochondrial aspartate. AGC consists of two domains: the C-terminal domain containing the sequence features of the mitochondrial carrier family and the N-terminal domain protruding in the intermembrane space and containing four binding sites for Ca^{2+} (Palmieri L, et al., EMBO J. 2001;20, 5060-9). AGC enables mitochondrial oxidation of cytosolic NADH reducing equiva-

lents, as a component of the malate-aspartate shuttle, and provides cytosolic aspartate for urea synthesis in liver. In man, there are two AGC isoforms: AGC1, encoded by SLC25A12, which is highly expressed in central nervous system (CNS), hearth and skeletal muscles, and AGC2, encoded by SLC25A13, which is expressed in liver and in several other tissues, but its expression in CNS has to be investigated. We have described the first case of AGC1 deficiency in a child who showed severe hypotonia, arrested psychomotor development and seizures from the first months of age (Wibom R et al., N Engl J Med. 2009;361, 489-95). MRI of the brain revealed lack of myelination in the cerebral hemispheres but not in brainstem and cerebellum. No focal lesion or pathological signal was detected in the cortical gray matter or basal ganglia. Importantly, proton MRS of the patient's left basal ganglia, in the occipital midline and the frontal lobe showed a drastic reduction of N-acetyl aspartate (NAA) peak. Biochemical analysis of patient's muscle biopsies revealed normal mitochondrial respiratory chain enzyme activities. However, mitochondrial ATP production was drastically reduced in the presence of glutamate or glutamate and malate as substrates. Sequencing of patient's SLC25A12 revealed a homozygous c.1769A>G transition in exon 17, causing a Q590R missense mutation. The mutant form of AGC1 was expressed in E. coli, purified and reconstituted in liposomes where it was completely unable to transport aspartate or glutamate as compared to the wild-type recombinant AGC1 (Wibom R et al., N Engl J Med. 2009;361, 489-95). The clinical phenotype of the patient resembles that of Slc25a12 knockout mice that has previously been reported to consist of motor coordination deficits, impaired myelination in the CNS along with a striking deficit in the levels of aspartate and NAA (Jalil MA et al. J Biol Chem. 2005, 280, 31333-9). NAA produced in neurons is known to undergo transaxonal transfer to oligodendrocytes where it supplies acetyl groups for the synthesis of myelin lipids. This suggests that in neurons AGC1 affects aspartate efflux from mitochondria to the cytosol and that this efflux is essential for NAA formation and subsequent myelin synthesis.

ABSTRACT N. 110

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**ZEVIANI MASSIMO**	
Telethon grant N.	**GGP11011**	
Total budget €	**388.600**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

MITMED: A MULTICENTER CONSORTIUM FOR THE IDENTIFICATION AND CHARACTERIZATION OF NUCLEAR GENES RESPONSIBLE FOR HUMAN MITOCHONDRIAL DISORDERS

Ghezzi Daniele (1), Melchionda Laura (1), Diodato Daria (1), Invernizzi Federica (1), Lamantea Eleonora (1), Ferrero Ileana (3), Dallabona Cristina (3), Baruffini Enrico (3), Costa Rodolfo (4), Da Re Caterina (4), Zeviani Massimo (1,2)

(1) Unit of Molecular Neurogenetics, Foundation Istituto Neurologico "C. Besta", via Temolo 4, 20126, Milano, Italy, Tel. +39.02.23942630, Fax +39.02.23942619, E-mail: zeviani@istituto-besta.it
(2) Mitochondrial Biology Unit, Medical Research Council, Cambridge, UK
(3) Department of Genetics, Biology of Microrganisms, Anthropology and Evolution, University of Parma, Italy
(4) Department of Biology, University of Padova, Italy

Mitochondrial disorders strike >1 in 5,000 children and adults and predominantly affect the nervous system and skeletal muscle, causing substantial morbidity and premature death. The genetic and biochemical intricacy of mitochondrial bioenergetics explains the extreme heterogeneity of mitochondrial disorders, a formidable challenge for both diagnostic workup and treatment. In addition to mtDNA mutations, an increasing number of nuclear genes encoding mitochondrial proteins has been identified in association with mitochondrial encephalomyopathies. However, it is still not clear how cells become dysfunctional in response to a mitochondrial biochemical defect. Although bioenergetic failure associated with reduced ATP biosynthesis appear to be critical in individual or combined MRC-complex defects, other mechanisms are also likely to be involved, and are probably predominant in the pathogenesis of specific syndromes, such as alterations of cellular redox status, the production of reactive oxygen species, compromised Ca2+ homeostasis, dysregulation of the proton-motive force and its dissipation, and mitochondrial pathways of apoptosis. The tremendous clinical, biochemical and molecular heterogeneity of mitochondrial disorders makes virtually each member of the whole mitochondrial proteome a candidate for disease. As a result, only 40% of adult-onset disorders are currently diagnosed at the molecular level, and much lesser so in infantile syndromes. However, new technological and biocomputational tools offer the possibility of rapid and affordable analysis of the exome, i.e. the coding regions of all genes in single individuals or small families. Mitochondrial disease proteins can then be selected by exploiting predictive softwares, dedicated databases, and ex-vivo experiments. We have identified several new disease genes, each responsible of distinct defects of the respiratory chain, mtDNA metabolism, or both. Structural analysis has allowed us to dissect out the molecular consequences of the ablation or defects of these proteins, and their physical status in normal and disease conditions. To gain further insight on function and mechanism of disease, we have then created specific recombinant lines in yeast, flies, and mice. We will present the most recent discoveries, concerning the identification of new disease genes, and the elucidation of pathogenic mechanisms, achieved under the umbrella of the GGP1011 grant.

ABSTRACT N. 111

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**ZEVIANI MASSIMO**	
Telethon grant N.	**GPP10005**	
Total budget €	**1.235.000**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2010**

THERAPEUTIC STRATEGIES TO COMBAT MITOCHONDRIAL DISORDERS

Zeviani Massimo (1,5), Rizzuto Rosario (2), Bernardi Paolo (2), Scorrano Luca (3,4), Carelli Valerio (6), Viscomi Carlo (1), Di Meo Ivano (1), Cerutti Raffaele (1)

(1) Unit of Molecular Neurogenetics, Foundation Istituto Neurologico C. Besta, via Temolo 4, 20126, Milano, Italy, Tel. +39.02.23942630; Fax +39.02.23942619; E-mail: zeviani@istituto-besta.it
(2) Department of Biomedical Sciences University of Padua, Italy
(3) University of Geneva, Switzerland
(4) Dulbecco Telethon Institute, Padua, Italy
(5) MRC-MBU, Cambridge, UK
(6) University of Bologna, Italy

MitCare aims at finding effective treatment for mitochondrial disorders due to defects of oxidative phosphorylation (OXPHOS), by implementing strategies based on (i) by-passing OXPHOS deficiencies (ii) correcting specific biochemical defects, (iii) combating their pathological consequences in several cell and animal models, with the final outcome of (iv) implementing clinical trials on patients.
Not only OXPHOS impairment determines energy failure but also a host of additional effects including overproduction of reactive oxidant species (ROS) and other toxic compounds, activation of apoptosis and alteration of several homeostatic and signal pathways. Therefore, therapeutic strategies need to correct multiple levels of cell and tissue specific dysfunction. MitCare is organized in three workpackages (WP).
In WP1 we are exploiting an array of patient-derived cell lines with specific biochemical defects that have been characterized for mitobiogenesis, calcium signaling, autophagy, and ROS production. These cells have been tested with compounds tackling different homeostatic pathways of mitochondria, including antioxidants and redox regulators (i.e. idebenone), antiapoptotic agents (e.g. cyclosporine) and regulators of autophagy (e.g. rapamycin), and will undergo high throughput screening using the NIH library of compounds.
WP2 is exploiting several mouse models corresponding to specific human mitochondrial disorders to test the effects of activators of mitobiogenesis in ameliorating muscular and CNS phenotypes associated with specific OXPHOS gene defects. In particular, we have demonstrated that activators of PGC-1a, the master regulator of OXPHOS genes can correct the motor deficit of our recombinant models. In WP2, we have also successfully tested AAV-gene replacement and bone-marrow transplantation in mouse models of ethylmalonic encephalopathy and MNGIE, both characterized by the accumulation of toxic substances due to abnormalities in mitochondrial metabolism. Finally, in WP2 we have characterized the effects of overexpression, or ablation, of OPA1, a gene responsible of syndromic or non-syndromic dominant optic atrophy in humans. OPA1 is a multifaceted factor controlling shape of mitochondria, confinement of apoptogenic cytochrome c, and, possibly, stability and integrity of mtDNA. Preliminary results suggest that overexpression of OPA1 can protect tissues from a number of insults, including denervation atrophy. Contrariwise, OPA1-less conditional mice do

manifest optic atrophy, providing a model for therapeutic interventions, e.g. AAV-mediated gene replacement. In WP3, we are testing the efficacy of approved or experimental compounds targeting the same pathways as in WP1 and WP2 in patients with Leber's hereditary optic neuropathy, dominant optic atrophy and ethylmalonic encephalopathy. Validated therapies will then be extended to patients affected by other mitochondrial disorders.

ABSTRACT N. 112

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SICILIANO GABRIELE**	
Telethon grant N.	**GUP09004**	
Total budget €	**217.100**	
Centres: **11**	*Duration (yrs):* **2**	*Starting year:* **2010**

CONSTRUCTION OF A DATABASE FOR A NATION-WIDE ITALIAN COLLABORATIVE NETWORK OF MITOCHONDRIAL DISEASES

Siciliano Gabriele (1), Mancuso Michelangelo (1), Angelini Corrado (2), Bertini Enrico (3), Carelli Valerio (4), Comi Giacomo (5), Minetti Carlo (6), Moggio Maurizio (7), Mongini Tiziana (8), Servidei Serenella (9), Tonin Paola (10), Toscano Antonio (11), Uziel Graziella (12), Zeviani Massimo (13), Mitochondrial Network (14)

(1) Department of Neuroscience, University of Pisa, S. Chiara Hospital, via Roma 67, 56126 Pisa, Tel. 050.993046, Fax 050.554808; E-mail: g.siciliano@med.unipi.it
(2) Neurological Clinic, University of Padova and IRCCS S. Camillo, Venice
(3) Bambino Gesù Hospital, Rome
(4) Neurological Clinic, University of Bologna
(5) Neurology Unit, Fondazione, IRCCS Ca' Grande Ospedale Maggiore Policlinico, Milano, Dino Ferrari Centre, University of Milan
(6) Neuropediatric and Muscle Disorders Unit, University of Genoa and G. Gaslini Institute, Genoa
(7) Neuromuscular Unit, Fondazione, IRCCS Ca' Grande Ospedale Maggiore Policlinico, Milano, Dino Ferrari Centre, University of Milan
(8) Neurological Clinic, University of Turin
(9) Neurological Clinic, University of Rome
(10) Department of Neurological, Neuropsychologic, Morphologic and Motor Sciences, University of Verona
(11) Department of Neuroscience, University of Messina
(12) Child Neurology Unit, The Foundation "Carlo Besta" Institute of Neurology, IRCCS, Milan
(13) Unit of Molecular Neurogenetics, The Foundation "Carlo Besta" Institute of Neurology, IRCCS, Milan
(14) National Wide Italian Collaborative Network of Mitochondrial Diseases

In recent years, a flurry of epidemiological studies has confirmed the notion that mitochondrial disorders are among the most common genetic disorders and a major burden for society. However, in contrast to the extraordinary progress in our understanding of the biochemical and molecular bases, we are still extremely limited in our ability to treat these conditions. Small patient populations represent the major impediment to progress in research and care. The development of a web-based register of patients with mitochondrial disease is needed to better understand the phenotypes and the natural history of these diseases.
The project GUP09004 started on February 2010. Eleven centers with expertise on mitochondrial medicine have been involved. To date, we have collected more than 1160 patients, with both adulthood and childhood onset of the disease.
The network has reached the following goals: 1. Establishment of an Italian network of clinical centers with specific expertise; 2. Creation of a validated web-based database, harmonized with other European Databases and Networks; 3. Characterization of a big cohort of cases.
Our database allows many phenotype-based and genotype-based studies. Two examples are given:
-Phenotype-based approach: exercise intolerance in mitochondrial diseases. Fatigue and exercise intolerance are common symptoms of mitochondrial diseases, difficult to clinically assess. We observed that more of 20% of our patients complained of exercise intolerance. This symptom was more strongly associated with specific mutations (i.e., 3243A>G). CK levels were increased in ˜ 34% of the patients with exercise intolerance, not confirming the notion that CK are normal in the great majority of mitochondrial patients. Moreover, all the other myopathic signs included in our database (muscle pain, muscle wasting, eyelid ptosis/ophthalmoparesis, cardiomyopathy) were associated with exercise intolerance. Ragged red fibers and, especially, COX-negative fibers were more frequent in the subjects with exercise intolerance, whereas lactate levels could not predict the presence of exercise intolerance.
-Genotype-base approach: the 8344A>G mutation in our database. Myoclonic epilepsy with ragged-red fibers (MERRF) is a rare mitochondrial syndrome, mostly caused by the 8344A>G mitochondrial DNA mutation. Most of the previous studies have been based on single case/family reports or case series with only few patients. 42 patients carrying the 8344A>G mutation were identified in our database. The great majority of them did not had the full-blown MERRF syndrome. Myoclonus was present in one out of five patients, whereas myopathic signs and symptoms, generalized seizures, hearing loss, eyelid ptosis and multiple lipomatosis represented the most common clinical features. Some asymptomatic mutation carriers have also been observed. Myoclonus was more strictly associated with ataxia than generalized seizures in adult 8344A>G patients. Our results showed higher clinical heterogeneity of the 8344A>G mutation than commonly thought. Moreover, MERRF could be better defined as a myoclonic ataxia rather than a myoclonic epilepsy.
Large, multicenter studies are strongly needed to better characterize the clinical picture and natural history of these diseases, in order to identify some countermeasures (i.e. pharmacological, physical, or others) capable of benefit the patients suffering with these chronic, still incurable disorders.

M. Mancuso, Angelini C., Bertini E., Carelli V., Comi G., Minetti C., Moggio M., Mongini T., Servidei S., Tonin P., Toscano A., Uziel G., Zeviani M., Siciliano G. The Nation-wide Italian Collaborative Network of Mitochondrial Diseases. Fatigue and exercise intolerance in mitochondrial diseases. Literature revision and experience of the Italian Network of mitochondrial diseases. Neuromusc. Disorders, 2012 Dec;22 Suppl 3:S226-9.

ABSTRACT N. 113

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**BIANCHI VERA**	
Telethon grant N.	**GGP09019**	
Total budget €	**287.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

DEOXYNUCLEOTIDE POOL IMBALANCE, MITOCHONDRIAL DNA MAINTENANCE AND DISEASE

Ferraro Paola, Frangini Miriam, Franzolin Elisa, Miazzi Cristina, Rampazzo Chiara, Reichard Peter, Bianchi Vera

Department of Biology, University of Padova, Via Ugo Bassi 58B, 35131 Padova, Tel. +39.049.8276282, Fax +39.049.8276280, E-mail: vbianchi@bio.unipd.it

Correct concentrations of DNA precursors (dNTPs) are essential for genetic stability. Deficiency or imbalance of dNTPs increases mutation rates causing genetic diseases. Correct dNTP concentrations are maintained by a network of synthetic and catabolic enzymes that varies with cell proliferation. The high requirest for dNTPs during S phase is met by the R1/R2 isoform of ribonucleotide reductase (RNR) and cytosolic thymidine kinase. Outside S-phase and in differentiated cells the two enzymes are degraded, dNTP synthesis is strongly downregulated and depends on the constitutive activity of the R1/p53R2 isoform of RNR and on mitochondrial (mt) thymidine kinase (TK2) and deoxyguanosine kinase (dGK). In humans mutations in p53R2, TK2 or dGK cause severe mtDNA depletion syndromes (MDS) with characteristic tissue-specific traits.
In the present project we focussed on the regulation of dNTP synthesis in quiescent cells where p53R2, TK2 and dGK are at the forefront and addressed questions of the tissue-specific effects of their genetic inactivation. We employed fibroblasts isolated from patients with MDS caused by TK2 or p53R2 mutations, or cellular model systems (T-lymphoblasts and mouse myoblasts) to work on 6 subprojects: (1) the salvage pathway in human fibroblasts. In two lines of TK2-mutated fibroblasts a low residual enzyme activity suffices to maintain mtDNA during quiescence because wild-type fibroblasts contain a large excess of TK2. Cytosolic and mt enzymes of the purine salvage pathway participate in the phosphorylation of deoxyguanosine and arabinosyl guanine to their triphosphates; (2) p53R2 in human fibroblasts. In quiescent p53R2-mutant cells ribonucleotide reduction occurs at a low rate and p53R2 deficiency limits dNTP supply for DNA repair and mtDNA replication; (3) Mt deoxynucleotide synthesis in muscle. In purified C2C12 myotubes dNTP synthesis is profoundly modified. We downregulated individually p53R2, TK2 and dGK by siRNA transfection. Silencing of either mt kinase, but not of p53R2, caused a 50 % depletion of mtDNA and an unexpected decrease of all 4 dNTP pools independently of

the enzyme's substrate specificity; (4) Mt transporters for deoxynucleotides. We studied the in situ activity of the mt pyrimidine nucleotide carrier PNC1 by isotope-flow methodology and genetic manipulation; (5) A model for T-cell immunodeficiency. We mimicked in vitro the inactivation of purine nucleoside phosphorylase, a catabolic enzyme whose genetic inactivation produces a severe immunodeficiency with a suspected mt involvement; (6) A knock-out mouse lacking mt 5'-deoxynucleotidase (mdN). Exploratory experiments with ko and wt MEFs showed only marginal changes in mtDNA and dTTP pools in quiescent ko cells. Possibly the potent cytosolic deoxynucleotidase cdN compensates for the loss of mdN thereby preventing mt dNTP imbalance.

ABSTRACT N. 114

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**SALVIATI LEONARDO**	
Telethon grant N.	**GGP09207**	
Total budget €	**243.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

PATHOGENESIS AND THERAPY OF PRIMARY COENZYME Q DEFICIENCY

Doimo Mara (1), Desbats Maria Andrea (1), Trevisson Eva (1), Casarin Alberto (1), Pertegato Vanessa (1), Cerqua Cristina (1), Cogliati Sara (2), Rodriguez Hernandez Maria Angeles (3), Santos Ocana Carlos (3), Clarcke Cathy (4), Enriquez Jose Antonio (5), Scorrano Luca (2), Navas Placido (3), Salviati Leonardo (1)

(1) Clinical Genetics Unit, Department of woman and child health, University of Padova, Via Giustiniani 3, 35128, Padova, Tel +39.049.8211402, Fax +39.049.8211425, E-mail leonardo.salviati@unipd.it
(2) Venitian Institute of Molecular Medicine, Padova
(3) Department of Chemistry and Biochemistry, University of California, Los Angeles, CA, USA
(4) Universidad Pablo de Olavide, Sevilla, Spain
(5) Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain

The aim of our project was to study the pathogenesis of coenzyme Q deficiency (CoQ) and to investigate the possible therapeutic strategies for these disorders.
We have contributed to the identification of mutation in COQ6 in a cohort of children with steroid resistant nephrotic syndrome (SRNS). COQ6 encodes a mono-oxygenase which oxidizes the C5 carbon of the aromatic ring of CoQ. The human gene is ubiquitously expressed and produces at least two isoforms (a and b), both encoding mitochondrial proteins. Human COQ6 isoform a can complement S. Cerevisiae strains lacking the orthologous gene, while isoform b appears to be catalytically inactive, but it retains (at least partial) structural stability. Deleted yeast strains cannot grow on non-fermentable carbon sources, but after transformation with human COQ6 isoform a they recover growth, and in part also CoQ production. This feature was employed to validate the pathogenicity of all mutations identified in the cohort of patients with SRNS. In fact all mutations severely impact yeast growth in selective media. However these mutations appear to be hypomorphic (including a C-terminal frameshift allele) confirming the notion that a complete block of CoQ biosynthesis is incompatible with life. Interestingly patients seem to respond well to oral CoQ supplementation.
We have also discovered that haploinsufficiency of COQ4 may cause CoQ deficiency which is responsive to CoQ administration. This is the only COQ gene thus far that displays haploinsufficiency. Interestingly expression levels of the residual COQ4 allele correlate with the severity of the CoQ deficiency.
The product of COQ4 has a fundamental role in CoQ biosynthesis in yeast because it organizes a complex containing most of the COQ gene products. We have demonstrated that this complex is present also in mammalian cells and that it interacts with the supercomplexes of the respiratory chain (RC). This interaction is both physical and functional, since it is required for an efficient biosynthesis of coenzyme Q. We showed that conditions that abolish the formation of RC supercomplexes also impair CoQ biosynthesis. This provides an explanation to the frequent finding of CoQ deficiency secondary to defects of the mitochondrial respiratory chain, and provides a rational for CoQ treatment in these patients.
Next, we have completed the characterization of three other human COQ genes, COQ5, COQ10b, and ADCK2, required for CoQ biosynthesis or its regulation.
We have identified two patients (there is just a single case reported in the literature thus far) with cardiofaciocutaneous syndrome due to BRAF mutations who had secondary CoQ deficiency, who also benefited to CoQ supplementation.
Finally we have tested whether bezafibrate could improve CoQ biosynthesis in patients with primary deficiency, but failed to detect any effect in a cell model. Bezafibrate was however effective in cells of patients with defects in SCO2 and in other mitochondrial genes.

ABSTRACT N. 115

Telethon Research Projects - Neuromuscular Diseases		
Principal Investigator	**CONTE CAMERINO DIANA**	
Telethon grant N.	**GGP10101**	
Total budget €	**128.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

HEREDITARY CHLORIDE CHANNELOPATHIES OF SKELETAL MUSCLE AND KIDNEY: FROM GENOTYPE TO PHENOTYPE AND NOVEL PHARMACOTHERAPEUTICAL APPROACHES

Desaphy Jean-François, Liantonio Antonella, Pierno Sabata, De Bellis Michela, Imbrici Paola, Altamura Concetta, Dinardo Maria Maddalena, Camerino Giulia Maria, Scaramuzzi Antonia, Carbonara Roberta, Conte Camerino Diana

Dipartimento di Farmacia, Sezione di Farmacologia, Università di Bari, Via Orabona 4, 70125, Bari, Italy, Tel. +39.080.5442801, Fax +39.080.5442801, E-mail: diana.conte@uniba.it

The project is aimed at the identification of drugs for the treatment of patients suffering from chloride channelopathies, such as Myotonia Congenita (MC) affecting skeletal muscle (aim #1) and Bartter syndrome (BS) and salt-sensitive hypertension (SSH) affecting kidney (aim #2). MC and BS are due to mutations in CLCN1 and CLCNKB/BSND genes encoding chloride channels of the CLC family. Polymorphisms in CLCNKA/KB genes are associated with SSH. The pharmacotherapy of these diseases is purely symptomatic, while specific ClC channel ligands are dramatically lacking.
In aim#1, we characterized a total of 8 CLCN1 mutations linked to recessive MC. Chloride currents were studied using patch-clamp in HEK293 cells transfected with mutant hClC-1 channels. While the pathogenic mechanism of some mutations is elucidated, it remains unclear for others. To better define the genotype/phenotype relationship, the interaction between mutants found together in compound heterozygous patients is being studied in co-transfected cell models.
The carbonic anhydrase inhibitor acetazolamide (ACTZ) is used empirically by MC patients. We tested ACTZ on hClC-1 channels in HEK293 cells. In symmetrical [Cl-], the drug shifts the channel voltage dependence by -20 mV. Yet this effect is greatly reduced in low [Cl-]i condition, suggesting that ACTZ have limited effect on chloride currents in patho/physiologic conditions.
The sodium channel (NaCh) blocker mexiletine is today the first choice drug in MC, but there is a critical need for other antimyotonic drugs [Desaphy et al, Eur J Clin Pharmacol, 2012]. Part of our research is aimed at evaluating prompt-to-use and newly-synthesized NaCh blockers in vitro [Catalano et al, J Med Chem 2012; De Luca et al, Neuromuscul Disord 2012; Desaphy et al, Front Pharmacol 2012; De Bellis et al, Biophys J, in press; Desaphy et al, Mol Pharmacol revised]. Using a newly-created pharmacological rat model of MC for in vivo preclinical screening [Desaphy et al, Neuropharmacol 2013], we individuated a number of NaCh blockers with an antimyotonic efficacy up to 70 fold greater than mexiletine.
In aim#2, we explored the pharmacological profile of CLC-K/barttin channels expressed in HEK293 cells using patch-clamp. A rational drug design allowed us to identify a newly-synthesized benzofuran derivative (SRA-36) as a very efficacious blocker of CLC-Ka currents with a 4 microM affinity. Surprisingly, in contrast to what observed in amphibian oocytes, niflumic acid fails to increase CLC-Ka currents in HEK cells but produce an inhibitory effect in the 1-1000 microM range. The drugs are being tested on CLC-K mutants. In parallel, in vivo studies demonstrated that acute administration of benzofuran derivatives to rats produced diuretic effects and lowered systolic blood pressure (SBP) in normotensive rats [Liantonio et al, J Hypertens 2012]. Preliminary results also indicated that benzofuran derivatives reduced SBP in spontaneously hypertensive rats.

ABSTRACT N. 116

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**GASPARINI LAURA**	
Telethon grant N.	**GGP10184**	
Total budget €	**423.700**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2010**

CLINICAL, NEURORADIOLOGICAL AND MOLECULAR INVESTIGATION OF ADULT-ONSET AUTOSOMAL DOMINANT LEUKODYSTROPHY (ADLD): DISSECTION OF LAMIN B1-MEDIATED PATHOPHYSIOLOGICAL MECHANISMS IN CELLULAR AND MOUSE MODELS

Cortelli Pietro (2), Brusco Alfredo (3), Brussino Alessandro (3), Giorgio Elisa (3), Di Gregorio Eleonora (3), Capellari Sabina (2), Parchi Piero (2), Liguori Rocco (2), Lodi Raffaele (2), Vaula Giovanna (4), Contestabile Andrea (1), Denise Ferrera (1), Canale Claudio (1), Giacomini Caterina (1), Gasparini Laura (1)

(1) Istituto Italiano di Tecnologia, Dept of Neuroscience and Brain Technologies, Via Morego 30, 16163 Genova Tel. +39 010 71781519; Fax +39 010 71781230; E-mail: laura.gasparini@iit.it
(2) IRCCS Istituto delle Scienze Neurologiche di Bologna, UOC Clinica Neurologica, Ospedale Bellaria, University of Bologna, Dept. Of Biomedical and Neuromotor Sciences, Bologna
(3) University of Torino, Dept of Medical Sciences, Torino, Italy
(4) AO Città della Salute e della Scienza, Dept of Neurology, Torino, Italy

Autosomal dominant leukodistrophy (ADLD) is a rare, progressive and fatal genetic disease associated with overexpression of the nuclear protein Lamin B1 (LB1). We have collected 2 ADLD families with LB1 gene duplication (Fam-1 and Fam-2, identified by Partners 1 and 2, respectively), and a third with a variant ADLD (ADLD-TO), carrying a deletion upstream of the LB1 gene but no mutations or duplication.
We investigated the clinical phenotype of 3 affected patients from Fam-1. The clinical and autonomic symptomatology is broad. The autonomic dysfunction is mostly characterized by cardiovascular and skin noradrenergic failure with preserved cardiovagal function and cholinergic sweat gland innervation. The magnetic resonance longitudinal analysis is ongoing. In one Fam-2 patient and one healthy subject, we performed western blot analysis of cerebral protein extracts and confirmed that, in the ADLD patient, LB1 is overexpressed in the frontal, temporal, occipital cortices and cerebellar gray matter.
At the genetic level, using a custom aCGH assay centered on the LB1 gene, we defined the duplications of Fam-1 and Fam-2 at nucleotide level. Fam-1 carries a 324.3 kb duplication (126,040,794-126,365,469) and a 12-nucleotide insertion at breakpoint level. Fam-2 carries a 154.5 kb duplication (126,068,010- 126,221,779) and the breakpoints share a 2 bp microhomology. A non-homologous end-joining (NHEJ) mechanism is likely causative of the duplication, although fork-stalling template-switching (FoSTeS) may be involved in Fam-2.
In ADLD-TO, we did not detect any element with regulatory function among selected evolutionary conserved regions (ECRs) upstream to the deletion by dual-luciferase assay. However, circular chromosome conformation capture revealed four non-conserved regions, three of which bound LB1 promoter only in subjects carrying the deletion. Two of these regions, 1 within (for both patient and control) and 1 upstream of the deletion (specific for the patient only), had enhancer activity in a dual-luciferase assay.
We have established primary cultures of human skin fibroblasts from 3 ADLD patients with LB1 duplication, 1 LB1 duplication carrier, 6 non-carrier siblings, 1 ADLD-TO patient and 9 healthy subjects. Using confocal and atomic force microscopy we found that, in fibroblasts of symptomatic ADLD patients, LB1 upregulation is associated with nuclear morphological abnormalities and increased nuclear stiffness. To investigate the gene expression profile, we performed microarray analysis on mRNA extracted from primary human skin fibroblasts and whole blood of duplication carriers and non-carriers siblings. Data analysis is underway.
Finally, we generated a conditional EGFP-loxP-hLB1 transgenic mouse overexpressing human LB1 upon recombination by Cre. Examination of the initial 8 founders revealed low levels of EGFP protein expression only in the heart, lung and liver of some of them. Analysis of further founders is ongoing.

ABSTRACT N. 117

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**ORLACCHIO ANTONIO**	
Telethon grant N.	**GGP10121**	
Total budget €	**345.000**	
Centres: **4**	*Duration (yrs):* **2**	*Starting year:* **2010**

IDENTIFICATION OF NEW DISEASE-CAUSING GENES IN HEREDITARY SPASTIC PARAPLEGIA

Di Lullo Martina (1), Lombardi Federica (1), Carosi Laura (1,2), Mearini Marzia (1), Babalini Carla (1), D'Aloia Maria Michela (1), Montieri Pasqua (1), Gaudiello Fabrizio (1), Miele Marialuisa (1), Tessa Alessandra (3), Nesti Claudia (3), Pippucci Tommaso (5), Magini Pamela (5), Pereira Baptista Julia (5), Ciccone Roberto (6), Zuffardi Orsetta (6), Seri Marco (5), Santorelli Filippo Maria (3), Orlacchio Antonio (1,2)

(1) Laboratorio di Neurogenetica, CERC-IRCCS Santa Lucia, Via del Fosso di Fiorano 64, 00143 Roma, Tel. 06.501703308; Fax 06.501703312; E-mail: a.orlacchio@hsantalucia.it
(2) Dipartimento di Medicina dei Sistemi, Università di Roma "Tor Vergata", Roma
(3) Laboratorio di Medicina Molecolare e Neurogenetica delle Malattie Neurodegenerative, Dipartimento di Medicina Molecolare, IRCCS Stella Maris, Calambrone (Pisa)
(4) Istituto di Neuropsichiatria Infantile, Università di Pisa, Pisa
(5) Dipartimento di Scienze Mediche e Chirurgiche, Università di Bologna, Bologna
(6) Dipartimento di Medicina molecolare, Università di Pavia, Pavia

Hereditary spastic paraplegia (HSP) refers to a group of genetically heterogeneous neurodegenerative disorders characterized by insidiously progressive gait disturbance due to spasticity and weakness in the lower extremities. Although the estimated prevalence of HSP is about 1/100000 in Europe, these disorders represent important health problems because they cause progressive functional deterioration and handicap. Autosomal dominant (AD), autosomal recessive (AR), or more rarely X-linked inheritance has been reported. Roughly 50 HSP genes, denoted spastic gait genes (SPG) or loci, have been localized, but only half of them have been cloned. Thus, further heterogeneity is expected.
We plan to characterize clinically and molecularly families with undiagnosed HSP, map new genetic loci and identify new HSP genes by adopting novel molecular techniques, including array-Comparative Genomic hybridization (CGH) and exome resequencing analyses, and correlate the clinical spectrum with the genetic findings.
We used MotorChip, a customized CGH array designed for neuromuscular disorders, to identify gene deletions/duplications in a set of AD-HSP cases. We identified additional mutations in known genes associated with either AD-HSP or AR-HSP. We cloned RTN2, the SPG12 gene. Mutations in RTN2 were associated with early-onset pure HSP. We also demonstrated that the RTN2 gene product participates in a network of interactions among HSP proteins involved in ER shaping, further supporting the hypothesis that abnormal ER morphogenesis is a pathogenic mechanism in HSP.
In summary, our data expanded the list of variants associated with HSP, enlarged the spectrum of known genes, and further improved our knowledge on the related pathogenesis.
References:
1. Guidubaldi A et al: Novel mutations in SPG11 cause hereditary spastic paraplegia associated with early-onset levodopa-responsive Parkinsonism. Movement Disorders 2011, 26:553-6.
2. Orlacchio A et al: Late-onset hereditary spastic paraplegia with thin corpus callosum caused by a new SPG3A mutation. Journal of Neurology 2011, 258: 1361-63.
3. Montenegro G et al: Mutations in the ER-shaping protein reticulon 2 cause the axon-degenerative disorder hereditary spastic paraplegia type 12. The Journal of Clinical Investigation 2012, 122:538-44.

ABSTRACT N. 118

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**DAGA ANDREA**	
Telethon grant N.	**GGP11189**	
Total budget €	**306.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

MODELS OF ATLASTIN FUNCTION AND DYSFUNCTION

Daga Andrea, Andreazza Camilla, Rossetto Maria Giovanna, Misticoni Giulia

Istituto Scientifico E. Medea, Conegliano Research Center, 31015 Conegliano, (Treviso) Italy

The hereditary spastic paraplegias encompass a rare and diverse spectrum of disorders which are normally characterized by progressive spastic weakness of the lower extremities. Genetically, HSPs are classified into autosomal dominant, autosomal recessive and X-linked forms. Clinically, they are subdivided into 'uncomplicated' HSP, in which the spasticity occurs in isolation, or 'complicated' HSP, where the spasticity is accompanied by other neurological disturbances. Although they manifest as disorders of the muscles of the lower extremities, HSPs are caused by a neuronal defect, namely axonal degeneration of the neurons which innervate the affected regions. SPG3A is the second most common HSP and is caused by mutations in the atlastin gene. Studies on human atlastins had suggested that these dynamin-like GTPases may play a role in Golgi and/or ER morphogenesis. Our work in Drosophila has demonstrated that atlastin plays a crucial role in the biogenesis and maintenance of the endoplasmic reticulum by controlling the homotypic fusion of ER membranes.
During the first year of funding we obtained the following results
1. We made important progress on the understanding the mechanism of atlastin function. We found that a 3-helix bundle (3HB) within the middle domain of the protein is required for oligomerization. Moreover, GTP binding induces a conformational change that reorients the GTPase domain relative to the 3HB to permit self-association but the ability to hydrolyze GTP is required for full fusion, indicating that nucleotide binding and hydrolysis play distinct roles. Oligomerization of atlastin stimulates its ability to hydrolyze GTP and the energy released drives lipid bilayer merger. Finally, increasing the distance of atlastin complex formation from the membrane inhibits fusion suggesting that this distance is crucial for atlastin to promote fusion.
2. We have generated transgenic lines for all selected pathological mutations and produced the corresponding proteins in bacteria. We found that both in vivo and in vitro these pathological mutants behave as partial loss of function mutations but they do not display any dominant negative or neomorphic effects suggesting that SPG3A is caused by haploinsufficiency.
3. We have analyzed the in vitro activity of atlastin-1 after liposome reconstitution but have not been able to observe membrane fusion. Analysis of the other human atlastins is in progress.
4. Although we have not yet been able to initiate a genetic screen for atlastin interactors, we have made progress on the analysis of the interactions between atlastin and reticulon as well as a member of the REEP family of proteins. Mutations in both reticulon and REEP families members are responsible for other forms of hereditary spastic paraplegia. We found that reticulon and REEP are strong genetic antagonistic interactors of atlastin. Our studies on reticulon suggest that this protein is likely involved in mediating ER membrane fission and thus opposes in vivo the fusion activity of atlastin.

ABSTRACT N. 119

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**LIBERI GIORDANO**	
Telethon grant N.	**GGP08057**	
Total budget €	**235.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

MOLECULAR CHARACTERIZATION OF SEN1/SETX-CONTROLLED PATHWAYS DEFECTIVE IN THE AOA2 AND ASL4 NEURODEGENERATIVE SYNDROMES

Alzu Amaya (1), Bermejo Rodrigo (1), Begnis Martina (1), Lucca Chiara (1), Piccini Daniele (1), Carotenuto Walter (1), Saponaro Marco (1), Brambati Alessandra (1,2), Cocito Andrea (1), Foiani Marco (1,3), Liberi Giordano (1,2)

(1) The FIRC Institute of Molecular Oncology (IFOM) Foundation, Via Adamello, 16, 20139, Milano. At IGM-CNR: Tel. 0382.546364, E-mail: giordano.liberi@igm.cnr.it
(2) Istituto di Genetica Molecolare del Consiglio Nazionale delle Ricerche (IGM-CNR), Pavia
(3) DSBB-Università degli Studi di Milano, Milano

Mutations in Senataxin gene, which encodes for a evolutionarily conserved DNA/RNA helicase, cause two severe neurodegenerative disorders, the Ataxia with oculomotor apraxia type 2 (AOA2) and juvenile Amyotrophic lateral sclerosis type 4 (ALS4).
Combining genomic and genetic approaches together with the analysis of replication intermediates, we unmasked a key role for budding yeast Senataxin in coordinating replication with transcription (Alzu et al., 2012. Cell). We show that the Senataxin protein associates with replication forks. Senataxin-deficient cells accumulate aberrant replication intermediates and DNA-RNA hybrids while forks clash head-on with RNA polymerase II (RNAPII) transcription units. These replication defects correlate with accumulation of DNA damage signals and genome instability in Senataxin mutants. Altogether our data suggest that Senataxin is recruited at replication forks and, by removing DNA-RNA hybrids that accumulate in transcription-replication collisions, prevents fork instability and DNA damage across RNAPII transcribed units. We also show that Senataxin-deficient cells are resistant to drugs that interfere with microtubule assembly, suggesting that senataxin-dysfunctions affect multiple aspects of cellular metabolism.
Together our data provide a new framework for understanding the pathological molecular mechanisms caused by Senataxin deficiencies.

ABSTRACT N. 120

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**TESTI ROBERTO**	
Telethon grant N.	**GGP11102**	
Total budget €	**373.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

INVESTIGATING NEW THERAPEUTIC APPROACHES TO FRIEDREICH'S ATAXIA

Rufini Alessandra, Fortuni Silvia, Arcuri Gaetano, Serio Dario, Cavallo Francesca, Benini Monica, Malisan Florence, Condò Ivano, Testi Roberto

Laboratory of Signal Transduction, Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy, Tel. 06.72596503; Fax 06.72596505, E-mail: roberto.testi@uniroma2.it

Friedreich's Ataxia is a genetic neurodegenerative disease that is caused by low levels of expression of the mitochondrial protein frataxin. The severity of disease progression is critically correlated to the amount of residual frataxin. Current therapeutic approaches are therefore aimed at increasing frataxin levels. This can be in principle achieved either by increasing the transcription rate or by interfering with its degradation. We have previously shown that frataxin is degraded through the ubiquitin/proteasome pathway and we have identified K147 as the single lysine on frataxin that is responsible for its ubiquitination and degradation. Importantly, we have described the therapeutic potential of the use of small molecules that interfere with frataxin ubiquitination pathway to prevent its degradation and increase its levels in FRDA cells [Rufini A, et al. Preventing the ubiquitin-proteasome-dependent degradation of frataxin, the protein defective in Friedreich's ataxia. Hum Mol Genet. 2011 Apr 1; 20(7):1253-61]. These molecules were selected in silico for their ability to interact with the K147-harboring pocket on frataxin and were shown to be able to prevent frataxin ubiquitination and to increase functional frataxin levels in patients cells. The aim of our current studies is the identification of new and more effective compounds that prevent frataxin ubiquitination. In an iterative process, molecules are selected from databases of millions of commercially available chemical compounds, by their docking to the frataxin K147-surrounding pocket. Their efficacy is then validated by testing their ability to prevent ubiquitination and upregulate frataxin in living cells. The data collected by this selection process allowed the identification of molecular determinants that confer activity to the selected compounds and are leading to the design of new molecules. These new leads are currently being validated in cell lines and will then be tested in murine animal models.
Another attractive therapeutic strategy to increase frataxin levels is the inhibition of the enzyme that specifically ubiquitinates frataxin. We are therefore pursuing the identification of the E3 ligase responsible for frataxin ubiquitination. We have performed a siRNA-based functional screening using a commercially available E3 ligase-restricted siRNA library targeting about 600 different E3 ligases. The system is based on the use of a frataxin-prolabel fusion construct, which, when transfected into cells, generates a luminescence signal that is proportional to the amount of frataxin expressed in the cells. From this screening we selected a few potential candidates that will now be validated in different cellular systems.

ABSTRACT N. 121

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**TARONI FRANCO**	
Telethon grant N.	**GGP09301**	
Total budget €	**465.700**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2009**

THE ROLE OF THE MITOCHONDRIAL m-AAA PROTEASE COMPLEX IN THE PATHOGENESIS OF HEREDITARY SPINOCEREBELLAR DEGENERATIONS

Di Bella Daniela (1), Magri Stefania (1), Fracasso Valentina (1), Plumari Massimo (1), Granata Magda (2), Muzi Falconi Marco (2), Taroni Franco (1)

(1) S.O. Genetica delle Malattie Neurodegenerative e Metaboliche, Fondazione IRCCS Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Tel. +39.02.23944580, Fax +39.02.700548648, E-mail: taroni.f@istituto-besta.it
(2) Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano

Autosomal dominant spinocerebellar ataxias (SCA) are a heterogeneous group of neurological disorders characterized by cerebellar dysfunction mostly due to Purkinje cell degeneration.
We have discovered that heterozygous AFG3L2 (ATPase family gene 3-like 2) mutations cause SCA type 28. Along with paraplegin, AFG3L2 is a component of the mitochondrial m-AAA complex, an evolutionarily conserved protease involved in protein quality control and protein maturation. The project is aimed at defining the molecular bases of SCA28 and elucidating the pathomechanisms underlying this disease and the distinct recessively-inherited spastic paraplegia (SPG7) caused by mutations in the AFG3L2 partner paraplegin. The different neurodegenerative phenotypes caused by these two proteins suggests that, despite their partnership, they may play distinct, possibly independent, roles.
AFG3L2 protein and transcript were found to be highly and selectively expressed in cerebellar Purkinje cells. Mutation analysis in a large cohort of patients demonstrated that AFG3L2 mutations account for ~3% of SCA with unknown defect. Functional analysis in an m-AAA-deficient yeast cellular model (yta10del/yta12del) demonstrated that the mutations (>20) located in the ATPase or in the protease functional domains of the protein cause respiratory deficiency and defective processing of m-AAA substrates. In a first group of mutants, co-expression of paraplegin does not rescue the defective phenotype, while co-expression of AFG3L2-WT results in reduced growth rate, thus indicating a dominant-negative mechanism for the mutation. Interestingly, the majority of mutations were found to be "paraplegin-responsive" as paraplegin co-expression rescues the defective phenotype. In these cases, the mechanism is likely to be haploinsufficiency or a weak dominant-negative effect, which might result in variably reduced penetrance and/or expressivity in affected people. We then investigated the possible coinheritance of AFG3L2 and SPG7 mutations in patients with spinocerebellar syndromes and identified 3 probands with functionally-relevant heterozygous mutations in both genes. Our data indicate that concurrent mutations in both components of the mitochondrial m-AAA complex may result in a complex phenotype, thus expanding the clinical spectrum of AFG3L2-associated mutations.
To better understand the role of m-AAA complex in the pathogenesis of SCA28, we performed genetic and biochemical screening in yta10del/yta12del yeast strains which overexpress normal or mutant human AFG3L2. yta10del/yta12del/afg3l2E691K yeast cells were transformed with a multicopy yeast genomic library and selected for suppression of the mitochondrial function defect. This strategy would allow to identify proteins which may regulate m-AAA activity and expression, perform a related function, or determine bypass pathways. Forty clones were isolated from 12,000 transformants and 16 of them were further validated for suppressor activity. Sequence analysis identified 10 interesting putative candidates. Although further analyses are needed to understand the mechanism of suppression, all the identified candidates perform functions relevant to mitochondrial metabolism and homeostasis.

[Di Bella D, et al. Mutations in the mitochondrial protease gene AFG3L2 cause dominant hereditary ataxia SCA28. Nat Genet 2010; 42:313-321].

ABSTRACT N. 122

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BRUSCO ALFREDO**	
Telethon grant N.	**GGP12217**	
Total budget €	**322.800**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2012**

SPINOCEREBELLAR ATAXIA TYPE 28: CELLULAR AND ANIMAL MODELS TO UNRAVEL THE PATHOGENESIS AND TO IDENTIFY POTENTIAL THERAPEUTIC TARGETS

Mancini Cecilia (1), Hoxha Eriola (2), Roncaglia Paola (3), Brussino Alessandro (1), Cagnoli Claudia (1), Lo Buono Nicola (1), Funaro Ada (1), Altruda Fiorella (4), Turco Emilia (4), Porcelli Anna Maria (5), Gustincich Stefano (6), Gasparre Giuseppe (7), Tempia Filippo (2), Brusco Alfredo (1)

(1) University of Turin, Dept. of Medical Sciences, via Santena 19, 10126 Torino, Tel. +39.011.6334480, E-mail: alfredo.brusco@unito.it
(2) Dept. of Neuroscience, National Institute of Neuroscience-Italy (INN) University of Turin
(3) European Bioinformatics Institute, Cambridge,United Kingdom
(4) Dept. of Molecular Biotechnology and Life Sciences, University of Turin
(5) Dipartimento di Biologia Ev. Sp., Università di Bologna
(6) Neurobiology Sector, SISSA/ISAS, Trieste, Italy
(7) Dipartimento di Scienze ginecologiche, ostetriche e pediatriche, Università di Bologna

SCA28 is an autosomal dominant ataxia with oculomotor anomalies, associated with AFG3L2 gene missense mutations. The encoded protein is a member of the mitochondrial m-AAA protease (ATPases Associated with a variety of cellular Activities), and forms an hexameric complex in the Inner Mitochondrial Membrane, which exerts proteolytic activity and protein quality surveillance functions.
We performed a whole genome expression profiling (based on Affymetrix Human Genome U133A 2.0 Chip Array) using lymphoblastoid cell lines (LCLs) from four SCA28 patients (mutations p.T654I, p.M666V, p. M666T and p.G671R), and six unrelated healthy controls matched for sex and age. We found 117 probes whose expression was statistically different, 60 of which up-regulated (Fold Change - FC = 1.5-16) and 57 down-regulated (FC = 0.7-0.1). The differentially expressed genes (n=76) were clustered, in five functional categories: (1) Cell Growth and Metabolism; (2) Apoptosis activation; (3) Oxidative Stress Response. To verify these pathways, we performed functional experiments on seven patients' LCLs which showed: 1) a delayed growth compared to controls ($p<0.001$), and an increased number of cells (> 15%) in G0/G1 phase($p<0.001$); 2) an increased mortality of patients' cells due to apoptosis (AnnexinV/Propidium Iodide test) ($p<0.05$); 3) increased lipid peroxidation in basal conditions ($p < 0.05$) and normal intracellular ROS levels (DCFH-DA test at FACS analysis). We also evaluated the respiratory chain activity in LCLs mitochondria, measuring ATP synthesis after treatment with specific respiratory chain blocking agents and total ATP production of LCLs. No significant difference compared to controls was measurable, likely because an impairment in ATP production is absent or below detectable levels. Finally, we did not find mitochondrial DNA (mtDNA) large deletions by long-range PCR or mtDNA copy number anomalies in SCA28 LCLs compared to controls.
In conclusion, whole genome expression profiling in SCA28 LCLs allowed to identify several altered pathways that may be related to the disease. It is conceivable that the activation of the G1-S checkpoint and apoptosis is due to a cell cycle checkpoint surveyor responding to an abnormal cellular stress coming from the mitochondria. In more sensible cell types or tissues, such as Purkinje cells in cerebellum, this may result in a detectable alteration leading to the disease.
We developed the knockin mouse model of SCA28 disease, expressing p.M665R, found in a mutation screening of European ADCA casistic (Cagnoli et al., 2010). Afg3l2M665R/+ mouse were generated thanks to a previous Telethon Grant and will be used in next months to elucidate important clues on SCA28 pathology: besides the characterization of the phenotype, mice will be used to obtain Mouse Embryonic Fibroblast (MEF) and Purkinje cells to clarify if LCLs features may characterize also tissue directly involved in SCA28 neurodegeneration.

ABSTRACT N. 123

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CASARI GIORGIO**	
Telethon grant N.	**GGP12235**	
Total budget €	**423.900**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

MITOCHONDRIAL DYNAMICS AND CALCIUM HOMEOSTASIS AT THE CROSSROAD OF THE AFG3L2-ASSOCIATED PATHWAY TO CEREBELLAR DEGENERATION. FROM MOLECULAR HYPOTHESIS TO PRECLINICAL TREATMENT

Maltecca Francesca, Cassina Laura, Consolato Francesco, Baseggio Elisa, Casari Giorgio

Università Vita-Salute San Raffaele e Ospedale San Raffaele, Via Olgettina 58, 20132 Milano. Tel +39.02.26433502, Fax +39.02.26436352, E-mail: casari.giorgio@hsr.it

Spinocerebellar ataxia type 28 (SCA28) is a novel form of juvenile-adult onset, slowly progressive, cerebellar ataxia characterized by unbalanced standing, gait incoordination, nystagmus, ophthalmoparesis and pyramidal signs. Several disease-causing mutations have been identified in the AFG3L2 gene. AFG3L2 is a mitochondrial protein that forms homo-oligomeric and hetero-oligomeric complexes with paraplegin in the inner mitochondrial membrane, named m-AAA proteases. These complexes are in charge of quality control of misfolded proteins and participate in the regulation of OPA1 proteolytic cleavage, required for mitochondrial fusion.
We characterized a mouse model of SCA28 that recapitulates the features of patients. In fact, it shows progressive ataxia due to degeneration and loss of Purkinje cells (PCs), the typical neuropathological hallmark of SCAs. We found that in SCA28 PCs undergo "dark degeneration" as they appear shrunk, atrophic and dark. This degeneration, which generally follows increased Ca2+ concentration associated to dysfunction of the glutamatergic system, is quite peculiar in SCA28, since it originates from mitochondrial dysfunction. We hypothesize that an inefficient Ca2+ internalization operated by damaged mitochondria is one of the early events in the pathogenesis of SCA28. This defect can increase Ca2+ concentration in PCs, thus triggering dark degeneration.
Recently obtained data support our pathogenetic hypothesis. Indeed, we found that mitochondria in which AFG3L2 is dysfunctional have decreased ability to internalize Ca2+. This defect is neither a consequence of global alteration in cellular Ca2+ homeostasis nor of the reduced driving force for Ca2+ internalization within mitochondria, since cytosolic Ca2+ transients and mitochondrial membrane potential remain unaffected. Moreover, experiments in permeabilized cells revealed unaltered mitochondrial Ca2+ uptake speed in Afg3l2-/- cells, indicating the presence of functional Ca2+ uptake machinery. Our results show that the defective Ca2+ handling in Afg3l2-/- cells is caused by fragmentation of the mitochondrial network, secondary to respiratory dysfunction and the consequent processing of OPA1. This leaves a number of mitochondria devoid of connections to the enoplasmic reticulum and thus without Ca2+ elevations, hampering the proper Ca2+ diffusion along the mitochondrial network. The recovery of mitochondrial fragmentation in Afg3l2-/- cells by overexpression of OPA1 rescues the impaired mitochondrial Ca2+ buffering, but fails to restore respiration. By linking mitochondrial morphology and Ca2+ homeostasis, these findings shed new light in the molecular mechanisms underlining neurodegeneration caused by AFG3L2 mutations.

ABSTRACT N. 124

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**DELIA DOMENICO**	
Telethon grant N.	**GGP10066**	
Total budget €	**218.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

INHERITED DEFECTS IN DNA DAMAGE SIGNALING CAUSING NEURONAL DEGENERATION: UNDERSTANDING THE MECHANISMS

Carlessi Luigi, Fusar Poli Elena, Delia Domenico

Fondazione IRCCS Istituto Nazionale Tumori, Department of Experimental Oncology, Via Amadeo 42, 20133 Milano, Italy, Tel +39.02.23902641, E-mail: domenico.delia@istitutotumori.mi.it

Human hereditary neurological disorders caused by mutations in genes involved in DNA damage response/repair (DDR) comprise around twenty entities, the most prominent being Ataxia-telangiectasia (A-T). A-T is a rare early-onset neurodegenerative syndrome caused by inactivation of the ATM protein kinase, which is essential for the initiation and propagation of the cellular response to DNA double-strand breaks (DSBs). ATM is also activated though Cys disulfide bond formation by oxidants, and in this context it acts as a sensor of reactive oxygen species (ROS) and promotes an anti-oxidant defence by protecting the cell from ROS accumulation. The neuropathogenesis of A-T could thus reflect a failure to respond to DNA damage and/or redox signalling.
Disease models are essential for unraveling the mechanisms underlying the neuropathology, but ATM knockout mice do not recapitulate the CNS phenotype. In this work we applied a reprogramming approach to generate a novel in vitro human A-T model in order to investigate the outcome of ATM ablation in neurons.
We derived iPSCs from the fibroblasts of A-T patients with premature truncating mutations in ATM, and from normal control fibroblasts by introducing the reprogramming factors Oct4, Sox2, Klf4 and c-Myc and from these we obtained expandable Nestin-positive neural progenitor cells (NPCs) via embryoid body formation and generation of neural rosettes. Following differentiation, NPCs gave rise to a high percentage of electrophysiologically active neurons expressing betaTubIII, MAP2 and GABA. Our results show that maximum Na+ current density is similar in CTRL and A-T cells. Also, base excision repair capacity, which is important for the removal of DNA single strand breaks caused by oxidative stress, appears to be normal in A-T cells. On the other hand, the reduced expression of p53, gammaH2AX, pSMC1 and pKap1 in irradiated A-T neurons indicates a clearly attenuated DDR in these cells. Moreover, mutant neurons display deficits in the expression of the pre- and postsynaptic markers Synaptophysin and PSD95 as well as of the neuronal growth-associated protein SCG10 and the K+ channel-interacting proteins KChIP.
This in vitro model represents a powerful new tool for exploring the mechanisms that cause degeneration of human ATM-deficient neurons and may also be exploited for detecting relevant therapeutic targets.

ABSTRACT N. 125

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**D'ADDA DI FAGAGNA FABRIZIO**	
Telethon grant N.	**GGP12059**	
Total budget €	**210.600**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

CONTROL OF ATM ACTIVITY BY DICER AND DROSHA RNA PRODUCTS

d'Adda di Fagagna Fabrizio (1,2)

(1) Telomeres and cellular senescence, Tel. 02.574303227, Fax 02.574303231, E-mail: fabrizio.dadda@ifom.eu
(2) Telomeres Lab, CNR Institute of Molecular Genetics Via Abbiategrasso 207, 27100 Pavia, Tel. +39 0382 5461

The DNA damage response (DDR) is a signaling pathway that arrests the proliferation of cells undergoing genotoxic events to preserve genome stability. ATM (Ataxia Telangiectasia Mutated) gene product is an apical kinase of DDR. DICER and DROSHA are crucial ribonucleases involved in RNA interference (RNAi). Components of RNAi are thought to have evolved to preserve genome stability from the attacks of viruses and mobile genetic elements. RNA products generated by DICER and DROSHA are involved in chromatin assembly, gene silencing and cancer. So far, RNAi and DDR pathways have not been demonstrated to directly interact.
We have recently shown in human, mouse and zebrafish that DICER and DROSHA, but not downstream elements of the RNAi pathway, are necessary to activate the DDR upon exogenous DNA damage and oncogene-induced genotoxic stress, as studied by DDR foci formation and by checkpoint assays. DDR foci are sensitive to RNase A treatment and DICER- and DROSHA-dependent RNA products are required to restore DDR foci in RNase-A-treated cells. Through RNA deep sequencing and the study of DDR activation at a single inducible DNA double-strand break, we demonstrate that DDR foci formation requires site-specific DICER- and DROSHA-dependent small RNAs, named DDRNAs, which act in a MRE11–RAD50–NBS1-complex-dependent manner to fuel DDR. DDRNAs, either chemically synthesized or in vitro generated by DICER cleavage, are sufficient to restore the DDR in RNase-A-treated cells, also in the ab-

sence of other cellular RNAs.
We propose an unanticipated direct role of a novel class of ncRNAs in the control of DDR activation at sites of DNA damage.

ABSTRACT N. 126

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**FOIANI MARCO**	
Telethon grant N.	**GGP12171**	
Total budget €	**331.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

MODEL SYSTEMS TO IDENTIFY GENES AND FACTORS IN THE SIGNAL TRANSDUCTION PATHWAY DEFECTIVE IN ATAXIA TELANGIECTASIA PATIENTS

Kumar Amit (1), Lucca Chiara (1), Pawan Singh (1), Ghadeer Shubassi (1), Michele Mazzanti (2), Marco Foiani (1,2)

(1) IFOM, Via Adamello 16, 20139 Milano, Tel. +39.02.574303238, E-mail: marco.foiani@ifom.eu
(2) Università degli Studi di Milano

Chromosome instability is hallmark of cancer cells and cellular aging. ATM and ATR protein kinases are apical DNA damage checkpoint proteins and regulate integrity of genome through coordination of the cell cycle progression with the DNA repair, DNA replication and recombination processes. Mutations in both ATR and ATM result in their dysfunction and are associated with autosomal recessive disorders Ataxia telangiectasia (A-T) and Seckel syndrome, respectively. The clinical manifestation of A-T and Seckel syndromes and the presence of ATM/ATR substrates outside of nucleus suggest their role in maintaining cellular homeostasis under stress and physiological conditions. We have recently suggested a role for ATR homologue Mec1 in maintenance of genomic stability through regulation of chromatin tethered regions at the nuclear envelope by phosphorylating nucleoporins. Our recent findings revealed that ATR localizes at the nuclear envelope under certain conditions. We tested the possibility that the topological tension caused by chromosome dynamics could induce mechanical stress at the level of nuclear envelope through those chromatin regions that are physically attached to the envelope. Hence, we extended our studies to vertebrates and examined whether the checkpoint kinase ATR is able to sense nuclear and plasma membrane tension following the induction of membrane stress using a variety of approaches, including mechanical stress on single cell. Our findings strongly suggest a link between ATR mediated nuclear events with the mechanotransduction occurring through the cytoskeleton connected to the plasma membrane of the cells.
Another feature that characterizes A-T patients is premature aging. We are therefore studying the activity of checkpoint proteins in aging cells, using budding yeast a model system. We found that, differently from young cells, old ones fail to activate the checkpoint and to repair UV induced DNA damage. We were able to partially restore the checkpoint response by treating cells with Rapamycin or Metformin, two TORC1-targeting compounds having a known effect on longevity. Moreover, these pharmacological treatments improve cells' ability to repair the DNA damage. Besides inducing DNA damage, UV treatment also causes protein damage, moreover damaged proteins are known to accumulate in cells as they age. One of the effects of targeting TORC1 activity is the modulation of protein synthesis, which can influence the cells' ability to respond to DNA damage. In agreement with the hypothesis that TORC1 pharmacological inhibition improves the DNA damage response by changing translation, we observed that mutations altering protein synthesis also have an effect on checkpoint activation and DNA damage repair in aging. We are now investigating the relative levels of factors implicated in the repair of UV induced lesions in new born and old cells.

ABSTRACT N. 127

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BENFENATI FABIO**	
Telethon grant N.	**GGP09134**	
Total budget €	**414.500**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2009**

INVOLVEMENT OF SYNAPSIN GENES IN EPILEPSY AND AUTISM

Lignani Gabriele (1), Fornasiero Eugenio (2), Greco Barbara (1), Fassio Anna (1), Baldelli Pietro (1), Leocani Letizia (2), Valtorta Flavia (2), Benfenati Fabio (1)

(1) Department of Experimental Medicine, University of Genova, Viale Benedetto XV n. 3, 16132 Genova, Italy, Tel. 010.3538183, Fax 010.3538194, E-mail: fabio.benfenati@iit.it
(2) Division of Neuroscience, San Raffaele Scientific Institute and Vita-Salute University, Milano, Italy

Goal of the project is the elucidation of the molecular mechanisms by which mutations in the synapsin (Syn) genes lead to the development of epileptic/autistic phenotypes and the evaluation of the physiological impact of the human SYN mutations associated with epilepsy and autism spectrum disorders (ASD).
1. Functional role of synapsin genes
To clarify the generation and expression of the epileptic phenotype, we performed patch-clamp recordings in the CA1 region of acute hippocampal slices. We found a strong imbalance between basal glutamatergic and GABAergic transmission with increased eEPSC and impaired eIPSC amplitude and a parallel derangement of short-term plasticity paradigms. A lower tonic GABAA current due to the impaired GABA release was found to be responsible for the more depolarized resting potential of SynI/II/III knockout (TKO) CA1 neurons, which makes them more susceptible to fire, and for the lack of the presynaptic GABAB receptor brake on glutamate release. In these TKO terminals, synaptic vesicles (SVs) are dispersed within the axonal lumen in both excitatory and inhibitory neurons, due to an increased fraction of mobile SVs. This higher SV mobility was not affected by stimulation, but was reverted by chronic neuronal activity blockade, indicating that Syns are essential to maintain the dynamic structural organization of synapses during intense SV recycling and that these changes could markedly impair inhibitory neurotransmission.
Electroencephalographic recordings of TKO mice showed spontaneous and evoked epileptic discharges with behavioral correlates, followed by a post-ictal phase characterized by a 4 Hz rhythmic activity, corresponding to immobility of the animal. Significant changes in background cortical activity with a slow-down of the high-frequency peak of EEG power spectra were also detected. Behaviorally, deletion of Syn isoforms impaired social behaviors, resulting in ASD-related phenotypes well before the onset of seizures. While all mutants displayed altered social behavior, SynII deletion strongly impaired various aspects of social memory and novel environment exploration and increased self-grooming. The results demonstrate an involvement of Syns in generation of the ASD behavioral traits and identify Syn KO mice as a useful experimental model of ASD and epilepsy.
2. Impact of the SYN1 mutations associated with epilepsy and ASD
Mutations in SYN1 are linked to idiopathic epilepsy and autism. We investigated synaptic transmission in mouse hippocampal SynI knockout neurons expressing the recently identified Q555X mutation. A decrease in the readily-releasable pool in inhibitory synapses and in the release probability in excitatory synapses caused a marked reduction of the evoked synchronous release. This effect was accompanied by an increase in asynchronous release that was much more intense in excitatory synapses. Q555X-hSynI induced larger facilitation and post-tetanic potentiation in excitatory synapses and stronger depression after long trains in inhibitory synapses. These changes were associated with higher network excitability and firing/bursting activity, recapitulating the KO phenotype. The data indicate that imbalances in short-term plasticity and release dynamics of inhibitory and excitatory synapses induced by the Syn mutations can trigger network hyperexcitability potentially leading to epilepsy/autism manifestations.

ABSTRACT N. 128

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CANCEDDA LAURA**	
Telethon grant N.	**GGP10135**	
Total budget €	**132.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

ROLE OF GABAA-RECEPTOR MUTATIONS IN IDIOPATHIC GENERALIZED EPILEPSY: A DEVELOPMENTAL STUDY

Szczurkowska Joanna (1), Cwetsch Andrzej (1), Fernandez Ignacio (1), Perlini Laura (1), Succol Francesca (1), Cossette Patrick (2), Benfenati Fabio (1), Barberis Andrea (1), Cancedda Laura (1)

(1) Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia (IIT), Via Morego 30, 16163 Genova, Italy, Tel. +39.010.71781521/751, Fax +39.010.71781230, E-mail: laura.cancedda@iit.it

(2) Neurologue, clinique d'épilepsie CHUM- Hôpital Notre-Dame, Montreal, Canada

Familial idiopathic generalized epilepsy (IGE) is a rare form of epilepsy associated with GABAA receptor (GABAAR) monogenic mutations. While functional analysis of the mutations provided in vitro evidence of a predictable decrease in cell inhibition that would favor an epileptic phenotype in vivo, other realistic scenarios are possible. In fact, GABAergic transmission plays an instrumental role in physiological neuronal-network development, and familial IGE patients express non-functional mutated GABAAR for life, thus also during brain development. We hypothesized that part of familial IGE phenotypes may depend on neuronal maldevelopment due to impaired GABAergic transmission.
Here, we investigated basic developmental mechanisms that may translate epileptic GABAAR defects into epilepsy phenotypes. In particular, we investigated whether defective GABAAR signaling during neuronal development in vitro influences the developmental switch of the temporal expression of distinct (alpha and delta) GABAAR subunit themselves. Furthermore, we developed an in vivo cortical model of rare familial IGE correlated to mutated gamma-subunit (the most abundant subunit of the GABAAR) by coupling RNA interference, which we developed against the gamma subunit, to in utero electroporation of the somatosensory cortex. Finally, we are also developing an in vivo cerebellar model of familial IGE correlated to mutated delta subunit of the GABAAR (highly expressed in the cerebellum) by the use of a new electroporation device, which we invented, that allows efficient genetic manipulation of the cerebellum. In these animal models, we have assessed neuronal migration and morpho/functional maturation of GABAAR-defective neurons. Next, we will investigate a possible correlation between the developmental defects and seizure propensity. This study will shed light on the possible adverse outcome of epileptogenic GABAAR mutations on neuronal development and possibly resulting epileptic phenotypes. Our experiments will expand the knowledge on the development of familial IGE, and may provide a window for therapeutic intervention before epileptic phenotype is overt.

ABSTRACT N. 129

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**NOBILE CARLO**	
Telethon grant N.	**GGP12078**	
Total budget €	**194.600**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

IDENTIFICATION OF NOVEL GENES FOR AUTOSOMAL DOMINANT LATERAL TEMPORAL EPILEPSY IN FAMILIES WITHOUT LGI1 MUTATIONS

Fanciulli Manuela (2), Dazzo Emanuela (1), Furlan Sandra (1), Michelucci Roberto (3), Nobile Carlo (1)

(1) Istituto di Neuroscienze del CNR, Sede di Padova, viale G. Colombo 3, 35121 Padova, Tel. 049.8276072; Fax 049.8276040; E-mail: nobile@bio.unipd.it
(2) Porto Conte Ricerche, Alghero
(3) Dipartimento Scienze Neurologiche, Ospedale Bellaria, Bologna

Autosomal dominant lateral temporal epilepsy (ADLTE) is a clinically homogeneous genetic syndrome characterized by focal seizures with prominent auditory or aphasic auras. Mutations causing ADLTE are identified in the LGI1 gene in less than 50% of families. Additional ADLTE-related genes are yet to be discovered to clarify the pathogenic mechanisms and for genetic counselling purposes. To search for novel ADLTE genes, we performed SNP-array copy number variation and linkage analyses of 16 LGI1-negative ADLTE families and mapped 3 linkage peaks with suggestive HLOD values (>2.0) on chromosomes 2p, 7q, and 19p. Other loci showing positive HLOD values were on chromosomes 4q and 11q. Overall, these results suggest that several other genes harbour mutations causing ADLTE, each underlying the syndrome in a small proportion of families. To identify these genes, whole exome sequencing appears to be the method of choice, though only a few examples of successful identification of genes involved in genetically heterogeneous Mendelian disorders have been reported. A major problem of exome sequencing is the large number of rare, potentially deleterious coding variants identified in each individual. In our study, the linkage data generated by SNP-array analysis will help us to overcome this problem by restricting the search for ADLTE-causative mutations to those regions with positive LOD scores in each family. We plan to re-sequence the whole exome in two affected members of each ADLTE family using the Agilent SureSelect human exon capture kit and Illumina HiSeq2000 sequencing apparatus. Common nonsynonymous, splice-site, and indel polymorphic variants already present in public databases will be filtered out, and rare nonsynonymous variants will be analyzed with programs such as SIFT to identify those more likely to be deleterious for proteins. Only the gene-variants found in both affected family members and, particularly, the very few lying in linkage peaks will be further investigated. In pilot experiments we performed on two of our ADLTE families, we found very few potentially pathogenic variants in two multiple-family linkage loci, each in a different gene. These genes are therefore excellent candidates for ADLTE and will be further studied. Overall, if a gene is found to harbour two or more deleterious heterozygous variants in different families, it will very likely be a causative gene for ADLTE, especially if one of such mutations results in protein truncation. If the function of a newly discovered ADLTE gene is already known, it will provide insights into the molecular mechanisms underlying ADLTE. Otherwise, preliminary biochemical and functional studies will be performed, which will depend on whether a newly identified ADLTE-related protein is secreted, is a receptor bound to the membrane or remains within the cell. Mutant proteins will also be assayed to determine the effects of epilepsy-causing mutations in vitro.

ABSTRACT N. 130

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BECCHETTI ANDREA**	
Telethon grant N.	**GGP12147**	
Total budget €	**179.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

THE ROLE OF NEURONAL NICOTINIC RECEPTORS IN THE PATHOGENESIS OF AUTOSOMAL DOMINANT NOCTURNAL FRONTAL LOBE EPILEPSY: A STUDY ON WILD-TYPE AND CONDITIONAL TRANSGENIC MICE EXPRESSING THE BETA2-V287L SUBUNIT

Meneghini Simone (2), Del Monte Noemi (2), Aracri Patrizia (2), Curia Giulia (5), Arcangeli Annarosa (3), Amadeo Alida (4), Becchetti Andrea (1)

(1) Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, 20126, Milano, Italy, Tel. 02.64483301; Fax 02.64483565; E-mail: andrea.becchetti@unimib.it
(2) Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca
(3) Dipartimento di Patologia e Oncologia Sperimentali e L.I.Ge.M.A. Università di Firenze
(4) Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano.
(5) Dipartimento di Scienze Biomediche, Università degli Studi di Modena e Reggio Emilia

OVERALL OBJECTIVES. We aim to understand the pathogenetic mechanisms of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and establish pre-clinical pharmacological models that may indicate how to prevent the epileptogenous process.
SPECIFIC AIMS. ADNFLE is often caused by mutations in heteromeric neuronal nicotinic acetylcholine receptors (nAChRs). We will study the relevant functional, anatomical, developmental and pharmacological aspects in transgenic (Tg) mice that conditionally express the beta2-V287L subunit (TET-off system), which we previously characterized in vitro.
BACKGROUND/RATIONALE. The pathogenesis of ADNFLE is unclear. Heterologous expression of mutant nAChRs suggests a gain of function, but it remains to be explained how focal human epilepsy can arise from hyperfunctional receptors expressed throughout the brain. Recent knock-in models indicate that the ADNFLE-linked mutations tend to alter neurotransmitter balance in the neocortex. The first conditional model of ADNFLE suggests that these effects may arise during neural development.
DESCRIPTION OF THE PROJECT. By modulating beta2-V287L expression, we will determine the sensitive stages for epileptogenesis. The epileptic phenotype will be tested in vivo by electrocorticography (ECoG). In parallel, we will determine the main electrophysiological and neuroanatomical changes induced by beta2-V287L expression, by using brain slices from the neocortex of adult animals. Regulation of neurotransmitter release and excitability will be mostly studied in layer V of prefrontal cortex. We will also study how beta2-V287L expression alters the excitatory/inhibitory transition of GABAergic transmission during development of the thalamocortical (TC) system, a likely mechanism for producing irreversible neural circuit alterations. Next, we will attempt to prevent the establishment of hyperexcitability via pharmacological treatment during the sensitive developmental stages.

ABSTRACT N. 131

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**FELLIN TOMMASO**	
Telethon grant N.	**GGP10138**	
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Centres: **5**	*Duration (yrs):* **3**	*Starting year:* **2010**

CELLULAR MECHANISMS UNDERLYING BRAIN DYSFUNCTION IN A MOUSE MODEL OF DRAVET SYNDROME

Scalmani Paolo (2), Farisello Pasqualina (1,3), De Stasi Angela Michela (1), Marcon Iacopo (4), Losi Gabriele (4), Cammarota Mario (4), Ratto Gian Michele (5), Arosio Daniele (6), Franceschetti Silvana (2), De Curtis Marco (2), Carmignoto Giorgio (4), Bacci Alberto (3,7), Mantegazza Massimo (8), Fellin Tommaso (1)

(1) Istituto Italiano di Tecnologia, Genova, Italia. - Dept. of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italia. Tel. 010.71781549; Fax 010.71781230; E-mail: tommaso.fellin@iit.it
(2) Fondazione IRCCS Istituto Neurologico C.Besta, Milano, Italia
(3) Fondazione EBRI "Rita Levi-Montalcini", Roma, Italia
(4) CNR-Istituto di Neuroscienze ed Università di Padova, Padova, Italia
(5) CNR- Istituto di Neuroscienze, Pisa, Italia
(6) Istituto di Biofisica, CNR, Trento, Italia
(7) ICM - Institut du Cerveau et de la Moelle épinière, Paris, France
(8) IPMC, CNRS UMR7275 and University of Nice-Sophia Antipolis, Valbonne, France

Mutations in the voltage-gated sodium channel NaV1.1 are linked to Dravet Syndrome (DS). DS mutations lead to loss in channel function and previous studies suggested that interneurons might be the main cellular population that is affected in DS. However, whether this effect is specific for given interneuronal subtypes and what might be the functional consequences of interneuronal dysfunction on synaptic networks is currently unknown. We have investigated these issues in NaV1.1 heterozygous knock out (NaV1.1-KO) animals, a mouse model of DS. Combining the use of acute hippocampal and cortical slices with patch-clamp recordings, we characterized the electrophysiological properties of different interneuronal subtypes. Recorded cells were characterized based on their electrophysiological properties and a posteriori morphological reconstruction or, in some experiments, interneurons were identified by using transgenic mice that expressed fluorescent reporters in specific interneuronal subtypes. Our results show that most hippocampal interneurons in strata oriens and lacunosum molecolare display a reduction in the discharge frequency in NaV1.1-KO compared to wild type (wt) animals. Analyzing the first suprathreshold action potential (AP), we found that the slope of the depolarization phase and the amplitude of the AP were significantly reduced in NaV1.1-KO with respect to wt. Preliminary results showed that different subtypes of basket cells in the pyramidal layer displayed a reduction in their firing properties in NaV1.1-KO mice. In cortical slices, we found that parvalbumin- and somatostatin-positive interneurons fire significantly less action potentials following current injections in NaV1.1-KO mice with respect to the same cells in wt littermates. These data suggest that the function of different interneuronal subtypes is affected in NaV1.1-KO mice, in contrast to what has been proposed in previous studies (i.e.: selective dysfunction of parvalbumin-positive interneurons). Recordings of spontaneous postsynaptic currents showed that the reduced interneuronal excitability is associated with decreased GABAergic synaptic transmission. Tonic GABAergic current is also reduced in NaV1.1-KO animals, in contrast to what observed in other animal models of epilepsy. We are currently analyzing whether these cellular modifications result in altered intracellular chloride concentrations. Network activities are also affected in Nav1.1-KO mice: the propagation of epileptiform events was found to be significantly faster in Nav1.1-KO compared to wt littermates and recurrent network oscillations in vivo displayed abnormal dynamics in Nav1.1-KO animals. Finally, combined video and EEG recordings during hyperthermia-induced seizures showed significant pre-ictal activity in Nav1.1-KO mice, particularly in the hippocampus. These results demonstrate that complex changes in the function of different interneuron subtypes may contribute to brain network alterations in DS.

ABSTRACT N. 132

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CARMIGNOTO GIORGIO**	
Telethon grant N.	**GGP12265**	
Total budget €	**446.800**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2012**

ROLE OF DYSREGULATED ASTROCYTE-GABAERGIC INTERNEURON INTERACTIONS IN THE CONTROL OF SEIZURES IN MONOGENIC MODELS OF EPILEPSY

Carmignoto Giorgio (1), de Curtis Marco (2), Ratto Gian Michele (3)

(1) Istituto di Neuroscienze del Consiglio Nazionale delle Ricerche - Viale Giuseppe Colombo 3, 35121, Padova, Tel. 049.8276075, Fax 049.8276040, E-mail: giorgio.carmignoto@bio.unipd.it
(2) Fondazione I.R.C.C.S. Istituto Neurologico "Carlo Besta", Milano
(3) Istituto di Nanoscienze, CNR NEST, Pisa

The mechanisms underlying the genesis and regulation of epileptic discharges are poorly defined. Our previous work supported by Telethon grant GGP07278 revealed that astroglial cells are capable of promoting neuronal synchrony and contribute to epileptiform discharges. These new findings redefine the basic principles that rule ictogenesis in epilepsy and are expected to change our understanding of seizure generation in acquired and genetic epilepsies.
Overall aim of the present grant is to clarify further the role of astrocytes in seizure generation. We believe that the GABAergic inhibition that controls seizure generation and propagation can be distinctively affected by astrocytes. The hypothesis that a dysregulated astrocyte-GABAergic interneuron signalling contributes to seizure generation will be investigated in two animal models of monogenic neurological diseases. In the model of severe myoclonic epilepsy of infancy due a loss-of-function mutation of a voltage-sensitive sodium channel (Nav1.1 KO mice), we will study the reciprocal signaling between astrocytes and GABAergic interneurons. In a model of familial hemiplegic migraine associated to seizures, due to an astrocytes-specific alteration of sodium-potassium ATPasea2 unit (Atp1a2 /R887 mice), we will analyze if seizures derive from defective uptake of glutamate and potassium. Ca2+ imaging at somata and astrocytic processes, glutamate and K+ measurements and neurophysiological recordings will be used in slices, in isolated in vitro brains and in vivo. Transgenic mice crossed with GCamp3 mice will be used to characterize the reciprocal signalling between astrocytes and interneurons/neurons during seizure generation and propagation. New expected data will significantly elucidate our understanding of the mechanisms underlying the genesis and regulation of epileptic discharges. Astrocytes have thus the potential to become primary targets for new therapeutic approaches in epilepsy.

ABSTRACT N. 133

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**NISTRI ANDREA**	
Telethon grant N.	**GGP10082**	
Total budget €	**221.900**	
Centres: **2**	*Duration (yrs):* **2**	*Starting year:* **2010**

STUDIES OF FAMILIAL HEMIPLEGIC MIGRAINE TRANSGENIC MOUSE MODELS AND PATIENTS TO INVESTIGATE THE CROSSTALK BETWEEN SENSORY NEURONS AND NEUROINFLAMMATORY CELLS IN TRIGEMINAL GANGLIA IN RELATION TO MIGRAINE PAIN

Ceruti Stefania (1), Fabbretti Elsa (4), Vilotti Sandra (4), Magni Giulia (1,2), Merli Davide (1), Verderio Claudia (3), Abbracchio Mariapia (1), Nistri Andrea (4)

(1) Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Dept. Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan
(2) Dept. Drug Discovery and Development, Italian Institute of Technology (IIT), Genoa
(3) CNR, Institute of Neuroscience, Milan
(4) International School for Advanced Studies (SISSA), Via Bonomea 265, 34136 Trieste, Italy, Tel. 040.3787718 Fax 040.3787249, E-mail: nistri@sissa.it

The main aim of the our research project is to understand what oc-

curs to pain transduction mechanisms of trigeminal sensory neurons in migraine, why they become hyperactive, and how we may stop them. Our project proposes to identify the chemical processes (including soluble messengers, with a specific focus on the purinergic system) used by trigeminal ganglion neurons to crosstalk with satellite glial cells (SGCs), and how this process might recruit neuroimmune cells to create a latent neuronal sensitization though which acute attacks are facilitated. To this end, we have employed tissues from a genetic mouse model of migraine, the Cav2.1 R192Q mutant knock-in (KI) mice expressing a human mutation causing familial hemiplegic migraine type 1 (FHM1).
Concerning the role of metabotropic purinergic receptors, our data show that application of bradykinin (BK) to primary mixed trigeminal cultures induces neuronal release of the pro-algogenic mediator calcitonin gene-related peptide (CGRP), which in turn potentiates the ADP-responsive P2Y1 and the UTP-sensitive P2Y2 receptor subtypes on surrounding SGCs (Ceruti et al., J Neurosci 31:3638-49, 2011). The increased activity of P2 receptors is not only due to increased receptor protein expression, but also, and especially for the P2Y1 subtype, to modulation of the receptor localization to membrane lipid rafts. In vivo studies on the possible pro- or anti-algogenic role of these receptor subtypes are currently in progress. Interestingly, the anti-migraine drug sumatriptan fully inhibits both CGRP release and glial P2Y-receptor potentiation. Moreover, exposure to BK leads to increased production of PGE2, an effect completely abolished by the COX-1 inhibitor acetylsalicylic acid. The latter also blocks neuronal CGRP release. Taken together, these results suggest a possible role for receptors activated by adenine and uracil nucleotides in the mechanism of action of currently employed anti-migraine drugs.
Unlike wild type (WT) trigeminal cultures, R192Q KI trigeminal ganglion cultures show basal macrophage activation together with enhanced TNFalpha release and neuronal currents mediated by P2X3 receptors. LPS application stimulates TNFalpha mRNA and WT P2X3 neuronal currents with faster recovery from desensitization (Franceschini et al. Purinergic Signal. 2012, E-pub July 27). Furthermore, co-culturing WT or KI ganglia with host macrophages strongly stimulate phagocytosis (Franceschini et al. BMC Neurosci 2012, Nov 21;13:143). These data suggest a basal neuroinflammatory profile in KI ganglia that facilitates the release of endogenous mediators (including ATP) to activate P2X3 receptors and amplify nociceptive signaling by trigeminal sensory neurons. The complex molecular cross-talk occurring even in basal conditions in KI ganglia further strengthen the role of purinergic receptors in the onset and maintenance of migraine-associated pain.

ABSTRACT N. 134

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BOLOGNESI MARTINO**	
Telethon grant N.	**GGP11057**	
Total budget €	**419.100**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE ROLE OF NEUROSERPIN IN FAMILIAL ENCEPHALOPATHY WITH NEUROSERPIN INCLUSION BODIES (FENIB)

Sessa Fabio (1), RIcagno Stefano (1), Bolognesi Martino (1), Manno Mauro (2), Martorana Vincenzo (2), Noto Rosina (2), Cupane Antonio (3), Levantino Matteo (3), Santangelo Maria Grazia (3), Miranda Elena (4), Lupo Giuseppe (4), Moriconi Claudia (4), Carucci Nicoletta (4), Timpano Valentina (4), Guadagno Noemi (4), Parisi Daniele (2,1)

(1) Dipartimento di Bioscienze, Università di Milano, Via Celoria 26, Milano. Tel. +39.02.50314893, Fax +39.02.50314895, E-mail: martino.bolognesi@unimi.it
(2) CNR Istituto di Biofisica, Palermo, Italia
(3) Dipartimento Fisica, Università di Palermo, Italia
(4) Dipartimento Biologia e Biotecnologie Charles Darwin, Università di Roma La Sapienza, Roma, Italia

Human neuroserpin (NS) belongs to the serpin family (serine protease inhibitor). NS is a glycosylated protein mostly expressed in neurons and it specifically inhibits tissue plasminogen activator (tPA). Six single mutations induce NS polymer accumulation leading to FENIB, a severe neurodegenerative disease, characterized by early onset dementia, epilepsy and neuronal death.
UNIMI unit: In order to study tPA inhibition, a stable NS:tPA complex was reconstituted in solution using inactive tPA mutants. Size exclusion chromatography (SEC) analysis revealed that the mutations did not affect the complex constitution and two different stable complexes have been isolated. In parallel we have also isolated NS latent form by SEC and, the crystallization of all these samples are currently under way.
Moreover, we are studying the polymerization mechanism using two different approaches. A) In order to pinpoint NS residues involved in polymerization, NS oligomers are being analyzed by cross-linking and the mass spectrometry. B) By contrast, to define the global architecture of the polymers we are combining crystallography with cryo-electron microscopy. For this purpose several specific antibodies able to recognize both the monomeric the polymeric forms are used. To date the crystallization of hNS:antibody complexes is under way.
IBF unit: We revealed the mechanism of NS polymerization in vitro. By scattering and chromatography studies, we have elicited the different processes involved (activation, dimerization, elongation, fragmentation, inactivation) and their peculiar role in the formation of NS polymers (Noto et al. 2012 PLoS ONE).
We have addressed the basic molecular aspects of NS functional and dysfunctional conformations. (i) A clear spectroscopic fingerprint for the different NS conformers have been identified by CD and PL spectroscopy, and preliminary results by EPR and site-directed spin labelling point to subtle differences in their dynamics and structure; (ii) the structure of NS polymers was shown to be context dependent by FTIR spectroscopy (Santangelo et al. 2012 Proteins); (iii) MD simulations have shown the existence of mechanically coherent domains allowing a local perturbation to affect the global NS dynamics.
UNIRO unit: We have focused on the cell biology of polymerogenic mutant variants of NS from two points of view: a) looking into the role that N-linked glycosylation has on NS's stability within the endoplasmic reticulum. Our results so far show that N-glycosylation is important for avoiding polymerisation, and that it can promote the degradation of mutant NS. b) We have developed a new cell model for the study of NS polymer toxicity, by transfection of wild type and disease causing variants of NS into neural stem cells from mouse cortex. The new system recapitulates the characteristics of the dementia FENIB in that mutant NS forms polymers within the endoplasmic reticulum and shows reduced secretion into the culture medium.

ABSTRACT N. 135

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**MATTEOLI MICHELA**	
Telethon grant N.	**GGP12115**	
Total budget €	**374.800**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2013**

MUTANT PRION PROTEIN IMPAIRS DELIVERY OF VOLTAGE GATED CALCIUM CHANNELS TO THE PRESYNAPTIC MEMBRANE: MECHANISMS OF NEUROTOXICITY AND POTENTIAL THERAPEUTIC STRATEGIES

Matteoli Michela (1,2), Senatore Assunta (3), Morini Raffaella (1,2), Ghirardini Elsa (1,2), Verderio Claudia (4), Restelli Elena (3), Pozzoli Manuela (3), Bertani Ilaria (3), Chiesa Roberto (3)

(1) Dip Biotecnologie Mediche e Medicina Traslazionale, via Vanvitelli 32, Universita' di Milano, Tel. 02.50317097, Fax 02.7490574, E-mail: michela.matteoli@unimi.it
(2) IRCCS Humanitas, Rozzano
(3) Dulbecco Telethon Institute and Mario Negri Institute for Pharmacological Research, Milano
(4) CNR Istituto di Neuroscienze, Milano

Broad objectives and specific aims
Evidence is emerging that neuronal loss in inherited prion diseases - incurable disorders of the CNS caused by mutations in the prion protein (PrP) gene - is preceded by synaptic dysfunctions, due to an adverse effect of misfolded PrP. We propose to clarify the mechanisms by which abnormal PrP affects normal synaptic activity, with the aim of devising effective strategies for early treatment.
Background and rationale
The proposal is based on our recent demonstration that mutant PrP is retained in the endoplasmic reticulum where it interacts with the Alpha2Delta-1 subunit of voltage gated calcium channels (VGCCs), thus impairing delivery of the channel complex to the cell surface. We plan to define how the defective trafficking of the channel complex is at the origin of neurodegeneration and whether this process can be functionally reversed.
Research design and methods
We will use an array of techniques and different transgenic models of prion disease to test the hypothesis that PrP misfolding leads to

synaptic defects and eventually to neuronal demise. More specifically, we will test whether the impaired VGCC delivery to the plasma membrane results in compensatory mechanisms increasing basal glutamate release, and whether trafficking defects also impact the correct localization and function of NMDA receptors, thus favoring excitoxic phenomena. We will also characterize the PrP- Alpha2Delta-1 interaction, with the outlook of devising strategies for disrupting mutant PrP- Alpha2Delta-1 association as a potential mean for therapeutic intervention.
Anticipated output
Defining whether defects in protein trafficking at the synapse originate excitotoxic phenomena is essential to identify novel molecular targets and to devise effective strategies for the therapy of inherited prion diseases, possibly taking advantage of the pharmacological tools already available in clinics.

ABSTRACT N. 136

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**FORLONI GIANLUIGI**	
Telethon grant N.	**GGP10208**	
Total budget €	**237.200**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

FATAL FAMILIAL INSOMNIA: PREVENTIVE TREATMENT WITH DOXYCYCLINE OF AT RISK INDIVIDUALS

Forloni Gianluigi (1), Tagliavini Fabrizio (2), Artuso Vladimiro (3), Redealli Veronica (2), Lucca Ugo (1), Tettamanti Mauro (1), Albanese Yasmin (1), Roiter Ignazio (3)

(1) Dipartimento di Neuroscienze, Istituto di Ricerche Farmacologiche "Mario Negri", Via La Masa 19, 20156 Milano, Italy Tel.02.39014462; Fax 0239001916
(2) Fondazione IRCCS - Istituto Neurologico "Carlo Besta", Milano, Italy
(3) Dipartmento di Medicina Interna, Unità Operativa di Medicina, Oderzo - ASL 9 Treviso ASL 9 Treviso, Italy

Fatal Familial Insomnia (FFI) is inherited disease in an autosomal dominant fashion and is linked to the aspartic acid to asparagine mutation at codon 178 of the prion protein gene in association with a methionine at the polymorphic codon 129 (D178N/M129). FFI is a rare genetic neurodegenerative disease characterized by disrupted sleep, autonomic hyperactivation and motor abnormalities. The disease is devastating and usually the patients die within two years from the onset and no treatments are available. In this project we carry out a preclinical treatment with doxycycline (DOXY) in subjects with a genetic risk to develop FFI. 85 members of a large FFI family were genotypized for the D178N mutation and some of them were found to carry the mutation. Based on the analysis of the previous cases (38 subjects) we determined in the range of 50-55 years of age the life period with highest risk to develop FFI. Since the penetrance is very high and the possibility to cure the disease after the clinical onset is remote, we propose a preventive treatment with DOXY to the carriers that were born between 1958 and 1969. The potential efficacy of DOXY in prion diseases derived from experimental investigations and two clinical observational studies in Italy and Germany with positive effects on survival and negligible side effects. Thus, in a double blind study, carriers receiving DOXY (100mg/die orally) will be matched with non-carriers belonging to the same family who will receive the placebo. To mantain blind the own genetic condition, also the subjects non-carriers will receive for a short period of time the DOXY treatment instead of placebo. Before starting the treatment and every second year afterward, all the individuals will be clinically examined. Although the size of the sample is limited, according to the statistical analysis we will be able within ten years to establish the efficacy of the treatment. This would be the first preventive study in FFI, which takes advantage of the unique opportunity to treat in a controlled condition a large pedigree of individuals at risk of developing the disease. The protocol of the study was finally approved by the Ethic Committees on December 2011 and on June 2012 the first subject enrolled has finished the clinical and neurological evaluation and started to receive the treatment. On November 2012, 10 subjects have concluded the initial clinical evaluation.

ABSTRACT N. 137

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**DI FEDE GIUSEPPE**	
Telethon grant N.	**GGP10120**	
Total budget €	**502.400**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2010**

BAD GENE, GOOD GENE: A RECESSIVE APP MUTATION CAN BE BOTH. NEW THERAPEUTIC PERSPECTIVE FOR ALZHEIMER'S DISEASE BASED ON AN ABETA VARIANT WITH DOMINANT-NEGATIVE EFFECT ON AMYLOIDOGENESIS

Diomede Luisa (2), Catania Marcella (1), Romeo Margherita (2), Morbin Michela (1), Palamara Luisa (1), Fugnanesi Valeria (1), Colombo Laura (2), Rossi Alessandro (2), La Rocca Paolo (2), Stoilova Tatiana (2), Salmona Mario (2), Tagliavini Fabrizio (1), Giuseppe Di Fede (1)

(1) U.O. Neurology V, Neuropathology, Fondazione IRCCS "Carlo Besta" Neurological Institute, Via Celoria 11, 20133 Milan, Italy, Tel. +39.02.23944648, +39.02.23944711, E-mail: gdifede@istituto-besta.it
(2) Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy

We have identified a novel mutation in Amyloid Precursor Protein gene (APP) resulting in A-to-V substitution at codon 673 [corresponding to the position 2 in amyloid protein (Abeta) sequence] which, in the homozygous state, results in a dominant role of Abeta1-40 in the aggregation pathway and composition of Abeta oligomeric assemblies and amyloid fibrils. Studies on biological samples from A673V homozygous carrier and cellular models indicated that this mutation shifts APP processing towards the amyloidogenic pathway with increased production of amyloidogenic peptides. Furthermore, the A2V substitution in the Abeta sequence (Abeta-A2V) increases the propensity of the peptides to adopt a beta-sheet structure, boosts in vitro and in vivo the formation of oligomers and enhances neurotoxicity. Following the observation that humans carrying the mutation in the heterozygous state do not develop Alzheimer's disease (AD), we carried out in vitro studies with synthetic peptides that revealed the extraordinary ability of AbetaA2V to interact with wild-type (WT) Abeta, interfering with its nucleation or nucleation-dependent polymerization. This offers a ground for the development of a disease-modifying therapy for sporadic AD based on modified Abeta peptides carrying the A2V substitution.
We designed a short peptide homologous to residues 1-6 of Abeta carrying the A2V substitution (Abeta1-6A2V) which retains in vitro the anti-amyloidogenic properties of the parent full-length mutated Abeta. The D-isomer [Abeta1-6A2V(D)], predicted to be more resistant to degradation by endogenous proteases, results even more effective than the L-isomer. To improve the translocation of the peptide across the blood brain barrier and the cellular membranes, an amino acid sequence highly rich in basic residues (TAT) has been linked to the six-mer peptide and the ability of Abeta1-6A2V-TAT(D) in inhibiting the oligomerization and neurotoxicity of WT full-length Abeta peptides has been investigated.
We tested the ability of this compound in inhibiting in vitro the fibrillogenic properties of the full-length Abeta and found that Abeta1-6A2V(D) hinders the production of fibrils and the generation of amyloid structures by the WT Abeta1-42.
Moreover, Abeta1-6A2V-TAT(D) hampers the toxicity induced by WT Abeta1-42 peptide on cultured SYSH-5Y neuroblastoma cells.
The transgenic C. elegans strain CL4176 expressing human Abeta1-42 in the body wall muscles, has been applied as simplified in vivo model to evaluate the efficacy of Abeta1-6A2V-TAT(D). In these nematodes the accumulation of Abeta1-42 in oligomeric forms is accompanied by the insurgence of the paralysis which is significantly counteracted by the treatment with the six-mer mutated TAT peptide.
All these findings indicate that the designed peptide is efficient in inhibiting WT Abeta polymerization and neurotoxicity. This anti-amyloidogenic ability will be ultimately tested in a mouse model of AD.

ABSTRACT N. 138

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**PROIETTI DE SANTIS LUCA**	
Telethon grant N.	**GGP11176**	
Total budget €	**94.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2011**

DISSECTING THE MOLECULAR BASIS OF NEURODEGENERATION IN COCKAYNE SYNDROME

Ciaffardini Flavia (1), Canu Giovanni (1), Balajee Adayabalam S. (2), Proietti-De-Santis Luca (1)

(1) Unità di Genetica Molecolare dell'Invecchiamento, Dipartimento di Ecologia e Biologia, Università della Tuscia, Largo Università, 01100 Viterbo, Tel. ++39.0761.357211, E-mail: proietti@unitus.it
(2) Center for Radiological Research, Department of Radiation Oncology, Columbia University Medical Center, New York, New York 10032, USA

Cockayne syndrome (CS) is a progressive developmental and neurodegenerative disorder that results in premature death during childhood. Most of the patients are mutated in the CSB gene. Although the genetic basis has been established for this disease, the precise molecular cause for neurodegeneration is not entirely clear. The present study was designed to verify whether or not CSB plays a critical role in survival, self-renewal and differentiation capabilities of neural stem/progenitor cells under normal and hypoxic conditions. Our innovative approach makes use of human neural progenitor cell system that is endowed with efficient self-renewal and multi-lineage differentiation capabilities. First we demonstrated that CSB knockdown in neural stem cells showed a dramatic reduction in survival and self-renewal capabilities, a feature which appears to be particularly enhanced under hypoxic condition. Further, CSB knockdown also dramatically reduced the differentiation potential of neural stem/progenitor cells. Along this line, neurite outgrowth, which is a characteristic feature of differentiating neurons, was dramatically affected in CSB silenced cells. Interestingly, CSB protein which is induced during neural differentiation, appear to play a double role during neuronal differentiation: in the nucleus it modulates that transcriptional programs that govern survival and differentiation of neural stem cells, second, in the cytoplasm it participates in the mechanism that orchestrate neurite outgrowth and retrograde axonal transport. Collectively, though currently in progress, this study suggests that CSB is a critical factor for protecting the functional integrity of neural stem/progenitor cells in self-renewal and differentiation processes.

ABSTRACT N. 139

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**DENTI MICHELA ALESSANDRA**	
Telethon grant N.	**GGP08244**	
Total budget €	**183.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

ANTISENSE RNA-INDUCED EXON-SKIPPING FOR THE GENE THERAPY OF FRONTOTEMPORAL DEMENTIA AND PARKINSONISM ASSOCIATED WITH CHROMOSOME 17 (FTDP-17)

Covello Giuseppina (1), Siva Kavitha (1), Lara Mari (2), Marchesi Elena (2), Perrone Daniela (2), Denti Michela Alessandra (1)

(1) Centre for Integrative Biology, University of Trento, via delle Regole, 101, 38123 Mattarello (TN), Italy, Tel.+39.0461.282740, Fax +39.0461.283091, E-mail: denti@science.unitn.it
(2) Dipartimento di Biologia ed Evoluzione, Università degli Studi di Ferrara

The neuropathological aggregation of microtubule (MT)-associated tau protein as Neurofibrillary Tangles (NFTs) in brain is a pathological hallmark of several neurodegenerative disorders, collectively known as "Tauopathies", among which Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17), a rare autosomal dominant condition. The genetic linkage between tau protein and FTDP-17 seems to be, for about half of the cases of FTDP-17, mutations affecting the alternative splicing of exon 10 (E10) of the tau mRNA. The aberrant inclusion of E10 in the tau mRNA, in fact, leads to the accumulation of the tau protein in neurons.
The project explores the feasibility of an antisense (as-) RNA-based gene therapy to correct tau splicing in FTDP-17.
We first tested whether it was possible to modulate E10 alternative splicing by the usage of Antisense Oligo-Nucleotides (AONs) that mask specific sites regulating splicing pathways.
RT-PCR and Western blot analyses showed that the transfection of specific AONs is able to alter the splicing behaviour of tau E10 in the rat endogenous transcript (PC-12 cell lines), with variable efficiencies depending on the concentration of the AONs and on the targeted sequence.
Based on these results, we constructed Adeno-Associated Viral (AAV) vectors coding for specific as-RNAs. We embedded the as-RNA sequences in chimeric U snRNA vectors whose promoters themselves lead to long-term as-RNA expression. We tested whether, the splicing behaviour of tau is corrected in endogenous rat mRNA by these chimeric antisense snRNAs.
To evaluate the effects of AONs/Chimeric Antisense-snRNA on the human tau pre-mRNA, we constructed a minigene reporter system, containing luciferase and that recapitulates to a large extent the behaviour of E10 in the context of the full-length tau gene. We carried out co-transfection into HeLa cells and evaluated the induction of E10 skipping by Luciferase Expression Assay and RT-PCR. Further work will be directed to test the therapeutic efficacy of the AAV-vectored as-RNAs in the animal model of FTDP-17 (T-279 mouse).

ABSTRACT N. 140

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CATTANEO ELENA**	
Telethon grant N.	**GGP12122**	
Total budget €	**192.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

IMPACT OF REDUCED GLIAL-DERIVED CHOLESTEROL IN HUNTINGTON'S DISEASE

Marullo Manuela* (1), Valenza Marta* (1), Di Paolo Eleonora (1), Cesana Elisabetta (2), Biella Gerardo (2), Cattaneo Elena (1)

(1) Centre for Stem Cell Research, Università degli Studi di Milano, via Balzaretti 9, 20133 Milano, Italy, Tel.02.50325840, Fax 02.50325843, E-mail: elena.cattaneo@unimi.it
(2) Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Pavia, Italy
* co-first-authors

Previous results have demonstrated reduced cholesterol biosynthesis in the brain of several Huntington's disease (HD) models. This dysfunction is manifest in astrocytes (Valenza et al., J. Neurosci. 2010). Brain cholesterol, which is synthesized locally, participates in several neuronal activities, such as neurite outgrowth and synaptic transmission. In post-natal life, cholesterol-dependent neuronal activities mainly rely on the transport of cholesterol from astrocytes on ApoE-containing particles.
We found that primary astrocytes from R6/2 HD mice and astrocytes from mouse neural stem (NS) cell lines that carried varying numbers of CAG repeats in the mouse Htt gene (Hdh50Q/7 and Hdh140Q/7 NS cells; Conforti et al., Neurobiol. Dis. 2012) have reduced cellular production and secretion of ApoE compared to their wild-type (WT) astrocytes. Our preliminary results also suggest that glia-conditioned medium (GCM) from WT astrocytes (GCMwt), or cholesterol administration, promotes neurite outgrowth and synaptic related parameters in primary and NS-derived HD neurons. Conversely, GCM from HD astrocytes (GCMHD), or GCMwt depleted of lipoproteins, fails to support neurite outgrowth and synaptic properties in HD neurons. Gain and loss of function experiments of key genes involved in cholesterol efflux and cholesterol synthesis in WT and HD astrocytes will further test the relevance of this cross-talk between astrocytes and neurons in normal and pathological conditions.

ABSTRACT N. 141

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**SQUITIERI FERDINANDO**	
Telethon grant N.	**GGP12218**	
Total budget €	**48.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

HD STAGE-DEPENDENT TGF-BETA1 PRODUCTION IN MACROPHAGES AND GLIAL CELLS

Di Pardo Alba (1), Alberti Silvia (1), Maglione Vittorio (1), Amico Enrico (1), Elifani Francesca (1), Concetti Fabio (1), Busceti Carla Letizia (2), Battaglia Giuseppe (2), Squitieri Ferdinando (1)

(1) Centre for Neurogenetics and Rare Diseases, IRCCS Neuromed, Pozzilli, Italy, Tel. +39.0865.915248, E-mail: squitieri@neuromed.it
(2) Neuropharmacology Unit, IRCCS Neuromed, Pozzilli, Italy

Huntington disease (HD) is the most common inherited neurodegenerative disorder caused by an expanded CAG repeat in the gene

encoding huntingtin (Htt). The neuropathological features of HD are linked to the disruption of multiple intracellular pathways that contribute to the development of the disease. Among others, defective production of neurotrophic factors may represent a possible determinant in HD pathology. Recently, we have reported a significant reduction of transforming growth factor-b1 (TGF-b1) levels since before disease onset in both nervous system (i.e. human post-mortem brain cortex) and periphery (i.e. serum) and argued that the defective bioavailability of the cytokine in early disease stage might contribute to the development of HD (Battaglia et al., J Cell Mol Med, 2011). In the present study we aimed to define which peripheral blood cell subset was primarily involved in the abnormal production of peripheral TGF-b1 in HD patients and whether changes of TGF-b1 levels during disease course might virtually reflect central defects and raise the possibility to identify a potential new biomarker of HD progression.

ABSTRACT N. 142

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**LEVI SONIA**	
Telethon grant N.	**GGP11088**	
Total budget €	**502.400**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE ROLE OF IRON AND MITOCHONDRIA IN THE PATHOGENESIS OF PANTOTHENATE KINASE ASSOCIATED NEURODEGENERATION (PKAN): DEVELOPMENT OF NEW NEURONAL CELLULAR SYSTEMS AND ANALYSIS OF A MOUSE MODEL

Campanella Alessandro (1), Brunetti Dario (2), Privitera Daniela (1), Santambrogio Paolo (3), Cozzi Anna (3), Guaraldo Michela (3), Garavaglia Barbara (2), Dusi Sabrina (2), Ruzzenenti Paola (4), De Luca Angela (4), Finazzi Dario (4), Tiranti Valeria (2), Levi Sonia (1)

(1) Università Vita-Salute San Raffaele, San Raffaele Scientific Institute, Division of Neuroscience, Via Olgettina 58, 20132 Milano, Tel. 02.26434755, Fax 02.26434844, E-mail: levi.sonia@hsr.it
(2) Unit of Molecular Neurogenetics, IRCCS Foundation Neurological Institute "C.Besta", Milano
(3) San Raffaele Scientific Institute, Division of Neuroscience, Milano, Italy
(4) Dipartimento Materno Infantile e Tecnologie Biomediche, Università di Brescia, Brescia, Italy

Pantothenate Kinase2 Associated Neurodegeneration (PKAN) is a genetic movement disease characterized by abnormal iron accumulation and degeneration in the brain basal ganglia. The Pantothenate Kinase2 (Pank2) gene responsible of PKAN encodes a mitochondrial key regulatory enzyme in Coenzyme A biosynthesis. The final goals of the project are to clarify the relationships between defects of Pank2 activity, brain iron deposition and mitochondrial dysfunction, and to test therapeutic approaches to the disorder. To this aims we first analyzed primary skin fibroblasts from three PKAN patients and from three controls. PKAN fibroblasts showed deficient control of the coordinate expression of TfR1 and ferritins in response to mild/long iron addition. This caused the increase of intracellular free iron, with a consequent iron-dependent ROS formation. Results suggested a role for iron in promoting oxidative stress and damages in PKAN cells (Campanella A et al. Skin fibroblasts from pantothenate kinase-associated neurodegeneration patients show altered cellular oxidative status and have defective iron-handling properties. Hum Mol Genet. 2012 Sep 15;21(18):4049-59). Dopaminergic neurons (iDAN) were obtained by patients' fibroblasts direct reprogramming by infection with Mash1-, Nurr1- and Lmx1a-lentivirus with an efficiency of ~5%, as identified by the expression of TuJ1, TH and N-CAM neuronal markers. Analysis at single cell level using DCF and TMRM showed an increment of ROS and a mild decrease of mitochondrial membrane potential in iDAN patients respect to the controls cells in basal condition.
In addition, we demonstrated mitochondrial localization of mouse Pank2 protein by western-blot analysis on sub-cellular fractions isolated from brain and fibroblasts of Pank2+/+ and Pank2-/- mice. We assessed that murine Pank2 protein is located in the mitochondrial inter-membrane space, probably anchored to the inner membrane (Brunetti D et al. Pantothenate kinase-associated neurodegeneration: altered mitochondria membrane potential and defective respiration in Pank2 knock-out mouse model. Hum Mol Genet. 2012 Oct 1. [Epub ahead of print]). Then, Pank2-deficent cells and null mice tissues were analyzed for iron homeostasis parameters, oxidative damage and mitochondrial functionality.
In HeLA and SH-SY5Y cells the perturbation of iron metabolism induced by Pank2 silencing is rescued by exposure to panthetine and is associated to an increased expression of other Pank isoforms. Ferritins evaluation in testis tissues from 4 Pank2-/- and 4 controls (2 Pank2+/+ and 2 Pank2+/-) mice indicate that FtL is ~1,6 fold higher in Pank2-/- mice. On the contrary the expression of the antioxidant proteins FtMt and SOD1 appears strongly decreased (~7 fold and ~2,5 fold respectively) in Pank2-/-respect to Pank2+/+ mice.
Microscale oxygraphy on mitochondria isolated from mouse brain demonstrated a defective respiratory profile in Pank2-/- as compared to Pank2+/+. Moreover, an alteration of mitochondrial membrane potential was detected, confirmed by electron microscopy in Pank2 -/- brains, peripheral nerve and neurons, showing the presence of swollen mitochondria with aberrant cristae and a complete modification of the matrix. These findings demonstrated that Pank2 deficiency causes alteration in iron homeostasis, oxidative damage and mitochondrial dysfunction in PKAN cells and animal models.

ABSTRACT N. 143

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**AROSIO PAOLO**	
Telethon grant N.	**GGP10099**	
Total budget €	**585.800**	
Centres: **5**	*Duration (yrs):* **3**	*Starting year:* **2010**

ANIMAL MODELS OF NEUROFERRITINOPATHIES FOR THE STUDY OF THE ROLE OF IRON IN NEURODEGENERATION

Cremona Ottavio (2), Giorgio Marco (3), Grohovaz Fabio (2), Levi Sonia (2), Arosio Paolo (1)

(1) Dipartimento Medicina Molecolare e Traslazionale, Università di Brescia, Viale Europa 11, 25129 Brescia, Italy, Tel. 0303717303, Fax 0303717305, E-mail: arosio@med.unibs.it
(2) Università Vita-Salute- San Raffaele Scientific Institute, Via Olgettina 58, 201232 Milano
(3) Dipartimento di Oncologia Sperimentale, Istituto Europeo di Oncologia, Milano

Neuroferritinopathies are rare genetic diseases with a dominant autosomal transmission caused by mutations in the C-terminus of ferritin L-chain (FTL). They belong to the Neurodegeneration with Brain Iron Accumulation (NBIA) group of pathology. We have studied the aging of animal models that express the pathogenic human FTL mutant 498InsTC (LN2) under the PGK promoter. Tg mice in the FVB background showed strong accumulation of LN2 in all tissues analyzed, particularly in the brain, that increased with aging, and this was accompanied by increased iron deposition detected by MRI, by the accumulation of ferritin-iron bodies detectable by Perl's stain and by signs of oxidative damage. Interestingly, the number of the iron bodies decreased after 3 weeks treatment with the oral iron chelator deferiprone. The mice then were backcrossed into the C57/BL6J background for 7 generations. Transgene ferritin accumulation was much lower in this background than in the FVB, and some LN2 mice showed sign of oxidative damage in the brain. Electron microscopy analysis revealed iron deposits associated with granular pigment in areas of cerebellum and striatum of a C57-LN2 mouse. These intracellular ultrastructures, never observed within nuclei, had a density one order of magnitude higher than in controls. Post-natal hippocampal neurons, obtained from C57-LN2 mice showed significant higher percentage of death in response to chronic iron overload and/or acute administration of H_2O_2, compared to control cells. Behavioral studies were initiated. The C57-LN2 mice tested at 2 months of age showed a defect in motor coordination (as measured through Beam Walking and Rotarod). Furthermore, we analyzed behavior in 2 year old LN2 (FVB background) treated with herbicides (Paraquat and Maneb) known to cause oxidative stress and neurodegeneration. No major motor deficit emerged, but the LN2 mice showed a paradoxical behavioural activation. We prepared vectors to generate new tg mice expressing human L ferritin (FTL-wt) and another pathogenic mutant (LN4). The sequences were optimized for expression in mouse, then cloned in a plasmid under the control of mouse PGK promoter and the constructs verified by sequencing. Experiments of transient transfection showed that the expression of the human ferritins in murine cells is considerably limited in respect with human cells. In another approach, recombinant pathogenic mutant heteropolymers were analyzed by cyclic voltammetry. This novel approach showed that the energetic of iron release of the mutant started much before that of WT, and that the mutant catalytically produced more ROS species in physiological solutions than wt. In conclusion, the transgenic mice recapitulate some of the pathological signs of human neuroferritinopathy, and are useful for testing new pharmacological strategies to reduce brain ferritin/iron accumulation.

ABSTRACT N. 144

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**VALENTE ENZA MARIA**	
Telethon grant N.	**GGP10140**	
Total budget €	**180.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

PINK1, MUTATED IN AUTOSOMAL RECESSIVE PARKINSON'S DISEASE, INTERACTS WITH THE PROAUTOPHAGIC PROTEIN BECLIN1 AND ITS ANTIAPOPTOTIC PARTNER BCL-XL: UNRAVELING THE SIGNIFICANCE OF THESE INTERACTIONS AT THE CROSSROAD OF MULTIPLE NEUROPROTECTIVE PATHWAYS

Arena Giuseppe (1,2), Gelmetti Vania (1), Torosantucci Liliana (1), Vignone Domenico (1), Cilia Emanuele (1), De Rosa Priscilla (1,2), Lamorte Giuseppe (1), Jonas Elizabeth (3), Valente Enza Maria (1,4)

(1) CSS-Mendel Institute, IRCCS Casa Sollievo della Sofferenza, viale Regina Margherita 261, 00198 Rome, Italy, Tel +39..416 0537, Fax +39.06.4416 0548, E-mail: e.valente@css-mendel.it
(2) Dept. of Experimental Medicine, Sapienza University, Rome, Italy
(3) Dept. of Internal Medicine, Yale University, New Haven (CT), USA
(4) Dept. of Medicine and Surgery, University of Salerno, Salerno, Italy

Mutations in PINK1 are a common cause of autosomal recessive Parkinson's disease (PD). PINK1 encodes a mitochondrial kinase implicated in maintaining mitochondrial homeostasis and function, preventing oxidative stress and apoptosis. In concurrence with Parkin, PINK1 regulates mitochondrial dynamics, trafficking and degradation through mitophagy. Moreover, PINK1 can activate autophagy by interacting with the pro-autophagic protein Beclin-1.
To further explore this pathway, we focused on members of the Beclin-1 complex, and found that PINK1 strongly interacted with Bcl-xL, a main anti-apoptotic protein of the Bcl-2 family also known to inhibit autophagy through Beclin1 binding. The interaction increased in cells treated with the mitochondrial uncoupler CCCP, that induces a robust accumulation of PINK1 at the outer membrane of depolarized mitochondria. In line with this, we showed by confocal microscopy that, upon CCCP exposure, Bcl-xL strongly co-localized with PINK1 wt but not with a mutant lacking the mitochondrial target sequence, suggesting that the PINK1-Bcl-xL binding mostly happens at the mitochondrial surface.
The interaction between PINK1 and Bcl-xL failed to disrupt the Bcl-xL-Beclin-1 binding; moreover, Bcl-xL was not required for Parkin recruitment and mitophagy activation. These findings indicated that the functional significance of the PINK1-Bcl-xL interaction is independent from the role of the PINK1-Parkin axis in regulating mitophagy.
We also showed that PINK1 was able to directly phosphorylate Bcl-xL both in vitro and in vivo, mainly at serine 62 (S62). The levels of phospho-Bcl-xL (p-Bcl-xL) increased with higher CCCP concentrations or longer time exposure, suggesting a direct correlation with the extent of mitochondrial depolarization. In this setting, PINK1 down-regulation or the over-expression of a kinase-defective mutant, dramatically reduced p-Bcl-xL levels.
By phosphorylating Bcl-xL S62, PINK1 significantly protected against CCCP-induced apoptosis, as revealed by the reduction of cleaved PARP levels and of apoptosis measured by FACS. Intriguingly, S62 is just adjacent to an aspartate (D61) that is cleaved upon apoptotic stimuli to generate a C-terminal pro-apoptotic fragment (deltaN-Bcl-xL). We showed that Bcl-xL cleavage was also triggered by mitochondrial depolarization, and the amount of deltaN-Bcl-xL strongly increased in the absence of PINK1. Cleaved Bcl-xL was significantly reduced in presence of the phospho-mimicking Bcl-xL S62E construct, suggesting that phospho-S62 could protect from CCCP-induced apoptosis by hampering the production of the deltaN-Bcl-xL fragment, for instance through a steric or electrostatic hindrance to the cleavage site.
Our data provide a functional link between PINK1, Bcl-xL and apoptosis, suggesting a novel mechanism through which PINK1 regulates cell survival. This pathway could be relevant for the pathogenesis of PD as well as other diseases including cancer.

ABSTRACT N. 145

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**GUSTINCICH STEFANO**	
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PERIPHERAL BLOOD GENE EXPRESSION PROFILING OF LRRK2 AND PARKIN MONOGENIC FORMS OF PARKINSON'S DISEASE FOR DISEASE ASSESSMENT

Calligaris Raffaella (1), Vlachouli Christina (1), Finaurini Sara (1), Vatta Paolo (1), Banica Mihaela (2), Tesolin Lucia (2), Cucca Alberto (2), Cattaruzza Tatiana (2), Antonutti Lucia (2), Goldwurm Stefano (3), Pizzolato Gilberto (2), Gustincich Stefano (1)

(1) Area of Neurobiology, International School for Advanced Studies (SISSA), via Bonomea 265, Trieste, Italy, Tel.+39.040.3787705, Fax +39.040.3787702, E-mail: gustinci@sissa.it
(2) Department of Clinical, Technological and Traslational Sciences, University of Trieste, Italy
(3) Parkinson Institute, Istituti Clinici di Perfezionamento, Milan, Italy

Parkinson's disease (PD) is a slow, progressive degenerative disorder of the central nervous system that is defined classically in terms of its motor symptomatology consequent to the selective degeneration of specific subsets of mesencephalic dopaminergic cells within Substantia Nigra (SN) pars compacta. Although dopaminergic drugs are clinically effective in alleviating motor symptoms in PD patients, no pharmacological treatment is currently available to slow or arrest the neurodegenerative process. At the onset of the motor symptoms, dopamine (DA) depletion in the putamen is 80% with 60% of dopaminergic neurons of the SN already lost, proving that PD is characterized by a long pre-symptomatic phase, lasting several years.
Numerous evidences indicate that PD is more than a disease of the nigrostriatal dopaminergic pathways, affecting diverse central and peripheral systems.
Blood transcriptomics is a powerful approach to analyze molecular processes of neurodegenerative diseases and to identify biomarkers as novel diagnostic tools.
PD aetiology is still largely unknown although studies concerning genes mutated in familial PD (fPD), such as LRRK2 and Parkin, have greatly contributed to the current understanding of the disease. However, it is unclear whether dominant and recessive forms of genetic cases present common mechanisms of neurodegeneration as well as their relation with sporadic PD (sPD).
Here we investigate the molecular events occurring in the blood of patients with LRRK2 and Parkin monogenic forms of Parkinson's disease. To this purpose, we have analyzed peripheral blood samples from patients with the dominant G2019S mutation in LRRK2 gene, from patients with inactivating mutations in Parkin gene and healthy controls by gene expression profiling.
We have thus identified genes whose expression are altered in fPD and compared them with those in sPD. Our ultimate goal is to provide new cues about the relationship between different forms of fPD as well as their commonalities and differences with sPD.
This may be important for the diagnosis of pre-symptomatic patients and for monitoring the effects of new therapeutic treatments

ABSTRACT N. 146

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**GOLDWURM STEFANO**	
Telethon grant N.	**GGP11164**	
Total budget €	**94.500**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2011**

IDENTIFICATION OF RECESSIVE GENES CAUSATIVE FOR PARKINSON'S DISEASE USING EXOME SEQUENCING

Goldwurm Stefano (1), Landers John (2), Duga Stefano (3)

(1) Parkinson Institute, Istituti Clinici di Perfezionamento, via Bignami 1, 20126 Milano, Tel. 02.57993319, Fax 02.57993468, E-mail: stefano.goldwurm@gmail.com
(2) University of Massachusetts Medical School, Worcester, Massachusetts, USA
(3) Department of Medical Biotechnology and Translational Medicine, University of Milan

This project aims to discover new genes involved in recessive

Mendelian forms of Parkinson Disease (PD) exploiting the recent development of exon capture and next-generation sequencing technologies.
The specific aims are: 1) to capture and sequence the exome of PD probands of recessive families (only affected siblings with consanguineous parents) and to select the potentially causative variants; 2) to verify if selected variants are specific for PD by analysing a large panel of cases/controls; 3) to search for mutations in the candidate genes in a group of potentially recessive PD cases; 4) to phenotypically characterize mutation carriers.
The identification of genetic causes of PD has greatly contributed to understanding the pathogenesis of this disease. Unfortunately, it is estimated that only a minority of the underlying genetic causes of familial PD have been explained to date. The recent development of exon capture and next-generation sequencing technologies now allows screening of cohorts for rare variants at a genome-wide scale in an economically feasible way.
To accomplish this goal, we focused on 10 PD patients from 7 families with consanguineous parents and 2 or more affected siblings. Such cases are highly suggestive of a recessive form of PD and represent a valuable resource for the identification of PD causative genes as their inbred nature overcomes several of the limitations that exist in the use of exome sequencing.
The exomes of these PD patients have been sequenced. All detected variants were filtered to select those present within coding regions and then tested to determine whether they were present in databases. Novel or rare variants (<5%) present in homozygous state in PD patients are currently tested in a large group (900) of controls subjects.
Variants absent in the control group are highly suggestive of a causative change. These selected variants will be tested in a large group of PD patients (familial and sporadic).
Anticipated output
We are confident that the proposed project will lead to the discovery of one or more novel causative genes for recessive PD. Such a result will shed new light on PD pathogenesis and identify new therapeutic targets likely valid in the more common complex forms of PD.

ABSTRACT N. 147

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CHIEREGATTI EVELINA**	
Telethon grant N.	**GGP10109**	
Total budget €	**187.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

PATHOGENETIC MECHANISMS OF FAMILIAL PARKINSON'S DISEASE: WT AND A30P ALPHA-SYNUCLEINS AFFECT THE STRUCTURE OF MICROFILAMENTS AND INTERMEDIATE FILAMENTS. PATHWAYS AND EFFECTS ON CYTOSKELETAL DYNAMICS

Emanuele Marco, Tilve Sharada, Difato Francesco, Ronzitti Giuseppe, Chieregatti Evelina

Dept. of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Tel. 010.71781740, Fax 010.71781230, E-mail: evelina.chieregatti@iit.it

Parkinson's disease (PD) is a progressive neurodegenerative disorder observed in approximately 1% of the population. It can be defined in biochemical terms as a dopamine-deficiency state resulting from degeneration of dopaminergic neurons, however the causes of neuronal cells death are still poorly understood. The strategy for treating PD has been to restore the dopamine deficit in the brain by pharmacological means or by neural grafting of dopamine-containing cells. Duplication, triplication and different mutations identified in the alpha-synuclein (Syn) gene have been found to cause familial early-onset PD, while genetic polymorphisms have been identified as risk factors for idiopathic PD. Throughout the brain, human Syn is highly expressed in nerve terminals where it plays a role in controlling neurotransmitter release. However, evidences that monomeric Syn is present in the cerebrospinal fluid and that healthy neuronal grafts transplanted in PD patients are often affected by Syn accumulation and degenerate, suggest that extracellularly secreted Syn may play a role in triggering the neurodegenerative process.
The goal of our work is to understand the effect of Syn on the functionality of the cytoskeletal system at the pre-synapse, which has a key role in neuronal activity and in neuronal regeneration. We showed that Syn and the A30P mutant affect microfilaments dynamics in opposite ways. Wt Syn decreases actin polymerization rate, whereas A30P Syn accelerates actin polymerization. Using the FRET technique, we are studying the subcellular localization and the regulation of Syn/actin association in cells and neurons. In cells transfected with the YFP-actin and CFP-Syn chimerae we observed an increase in the FRET efficiency that occurs specifically in a compartment close to the plasma membrane. In hippocampal neurons virally infected with the FRET pair, we show that the association between the two proteins increases as a consequence of neuronal stimulation by high KCl or by electrical field.
The cellular processes involved in neuronal development in the brain areas of adult neurogenesis, such as polarization, neurite elongation, and axon guidance are highly regulated by the actin cytoskeleton, as is axonal regeneration upon injury. In animal models of PD, adult neurogenesis is impaired and the inability of neurons to regenerate can aggravate the pathology. We established a dissector system that allowed us to perform axonal injury of mouse neurons at early days in culture with sub-cellular precision. After partial dissection, the reorganization of the cytoskeleton was documented by long-term live imaging. Upon axonal lesion, we found a decrease in the processes involved in actin dynamics, such as the number and velocity of actin waves, in neurons infected or incubated with wt and A30P Syn.
The understanding of Syn mechanisms of action on neurons functionality and development will be fundamental for the discovery of new therapeutic targets.

ABSTRACT N. 148

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**GREGGIO ELISA**	
Telethon grant N.	**GGP12237**	
Total budget €	**350.000**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2012**

FUNCTION AND DYSFUNCTION OF THE PARKINSON'S DISEASE KINASE LRRK2 AT THE PRE-SYNAPTIC SITE

Civiero Laura (1), Belluzzi Elisa (1), Cirnaru Maria Daniela (2), Russo Isabella (1), Marte Antonella (3), Pischedda Francesca (2), Longo Francesco (4), Bubacco Luigi (1), Morari Michele (4), Onofri Franco (3), Piccoli Giovanni (2), Greggio Elisa (1)

(1) Dipartimento di Biologia, Università degli Studi di Padova, Via Ugo Bassi 58B, 35121, Padova, Tel. +39.049.8276244, Fax +39.049.8276300; E-mail: elisa.greggio@unipd.it
(2) Istituto di Neuroscienze, IN-CNR, Milano
(3) Dipartimento di Medicina Sperimentale, Sezione di Fisiologia Umana, Università degli Studi di Genova
(4) Dipartimento di Medicina Clinica e Sperimentale, Sezione di Farmacologia, Università degli Studi di Ferrara

Mutations in Leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial Parkinson's disease (PD) but the pathogenic mechanisms that lead to disease are still unclear. LRRK2 is a large signaling/scaffolding molecule containing kinase and GTPase domains as well as protein-to-protein interaction motifs. Although the physiological function of LRRK2 is still unclear, we recently reported that LRRK2 modulates neurotransmitter release by orchestrating synaptic vesicles (SV) trafficking through interaction with different components of the secretory machinery. Synaptic dysfunction has been observed to contribute to the onset and progression of Parkinson's disease. We previously showed that the common G2019S mutation enhances kinase activity and that this activity is required for the neurotoxicity associated with mutant proteins. Based on these observations, our hypothesis is that mutant LRRK2 abnormally increases the phosphorylation levels of key pre-synaptic proteins, which leads to impairment of synaptic function and, in turn, neurodegeneration. Here we report our most recent observations. First, we found that the ErbB family epidermal growth factor receptor (EGFR) activates LRRK2. Treatment of LRRK2 expressing cells with EGF results in enhanced autophosphorylation, membrane-relocalization and stabilization of LRRK2. EGFR activation has been already reported to modulate presynaptic function. Thus not surprisingly we also found that LRRK2 efficiently interacts with isolated SV and actin filaments (which provide the track for SV movement) and phosphorylates key proteins involved in SV cycle, such as NSF and synapsin I. Furthermore, PAK6, a kinase associated with actin filaments, acts upstream of LRRK2 inducing its dephosphorylation and relocalization. Finally, LRRK2 silencing in cortical neurons significantly increases stimulus-evoked post-synaptic currents (EPSC) and SV mobility, whereas K+ stimulated striatal synaptosomes from LRRK2 knock-out mice display increased [3H]-dopamine release

compared to controls. Taken together, our biochemical and electrophysiological observations strongly indicate that LRRK2 is activated by EGFR cascades and orchestrates SV trafficking through interaction/phosphorylation with key pre-synaptic proteins. Targeting LRRK2 kinase activity or upstream components of the signaling cascade is a promising therapeutic strategy for PD.

ABSTRACT N. 149

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CICCONE ROBERTO**	
Telethon grant N.	**GGP08226**	
Total budget €	**384.800**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2008**

HIGH RESOLUTION ARRAY-CGH AND GENE EXPRESSION ANALYSES IN AUTISM SPECTRUM DISORDERS

Ciccone Roberto (1), Fichera Marco (2,3), Elisabetta Trabetti (4), Prandini Paola (4), Della Mina Erika (1), Bayindir Baran (1), Bulgheroni Sara (5), Franco Anna (6), Bonaglia Maria Clara (7), Di Benedetto Daniela (2), Musumeci Sabastiano Antonino (2), Giusto Stefania (2), Zusi Chiara (4), Malerba Giovanni (4), Xumerle Luciano (4), Politi Pierluigi (8), Brighenti Maurizio (6), Riva Daria (5), Orsetta Zuffardi (1)

(1) Dipartimento di Medicina Molecolare, Università di Pavia, Via Forlanini 14, 27100 Pavia, Tel. 0382 987726, Fax 0382 525030, E-mail roberto.ciccone@unipv.it
(2) IRCCS Associazione Oasi M.SS Troina, Italy
(3) Genetica Medica, Università di Catania, Catania, Italy
(4) Dept. Life and Reproduction Sciences, Section of Biology and Genetics, University of Verona, Verona, Italy
(5) UO Neurologia dello Sviluppo, IRCCS Istituto C. Besta, Milano, Italy
(6) Centro Diagnosi, Cura e Ricerca per l'Autismo, Verona, Italy
(7) Istituto Scientifico E. Medea, Bosisio Parini, Italy
(8) Dipartimento di Sanità Pubblica, Neuroscienze, Medicina Sperimentale e Forense, Università di Pavia, Pavia, Italy

Autism is characterized by impairment in social interactions, deficit in verbal and nonverbal communication, and repetitive and stereotyped patterns of behaviours, interests and activities. Autism is considered one of the most prevalent heritable complex diseases. Several evidences suggest that this disease has strong genetic bases but very few genes implicated in this disease have been identified. The aim of our project is to use high-resolution array-CGH technique to detect genomic alterations in patients with autism and to define their pathological role. Gene expression analysis on lymphoblastoid cell lines from these individuals is performed to figure out the pathological role of CNVs identified by array-CGH.
In total, 273 autistic patients were screened by high resolution array-CGH (180K Agilent). We identified 163 non polymorphic CNVs in 118 patients. The size of these variants was variable, ranging from 11 kilobases to 4.9 megabases; 88 were deletions, 75 were duplications. Each CNV was verified in the Autism Databases and compared with published data, allowing us to distinguish CNVs into those already described in autistic patients, and those that were not.
31 CNVs (18 deletions and 13 duplications) contained previously reported autism-related genes. Among these, 9 were de novo CNVs, 11 weren't investigated in parents, 11 were inherited from an apparently healthy parent. Among genes already linked to autism we identified imbalances for GRPR, CNTNAP2, NRXN1, SYNGAP1, AUTS2, DOCK8, DISC1, SOX11, CNTN4, ASMT, DOCK4 and NRXN3.We have also identified some non-polymorphic CNVs that we considered potentially causative of autism since some of these contained genes whose function/expression may suggest their involvement in autism aetiology.
Blood samples from autistic subjects and their families have been collected from four Italian neurology centres. After array-CGH analyses, 27 individuals carrying genomic imbalances, considered causative, have been analyzed for whole gene expression by next generation sequencing (RNA-Seq), to better understand the pathological role of the CNVs. Because brain tissue is not available, all gene expression experiments were carried out using mRNA from lymphoblastoid cell lines (LCLs). To assess which dysregulated genes could direct to pathogenic mutations, we investigated expression variance in each subject and identified genes, refer as outliers, with significant deviations in expression. To explore the functional impact of CNVs in ASDs at a genome-wide scale, our interrogation utilized the overlap between structural-variation data and transcriptional data. We demonstrate that two outlier genes (KIAA0430 and PKD1P6) cluster within a potentially involved CNV on chromosome 16 (already found in ASDs).

ABSTRACT N. 150

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CHERUBINI ENRICO**	
Telethon grant N.	**GGP11043**	
Total budget €	**385.600**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2011**

MECHANISMS UNDERLYING ALTERED GABAERGIC SIGNALING IN THE HIPPOCAMPUS OF TRANSGENIC MICE CARRYING THE HUMAN R451C MUTATION OF THE NLG3 GENE: AN ANIMAL MODEL OF AUTISM

Barberis Andrea (2), Cellot Giada (1), Cherubini Enrico (1), Petrini Enrica (2), Pizzarelli Rocco (1), Ruggeri Federica (1), Stancheva Stefka (2), Zacchi Paola (1)

(1) Neurosciece Dept., International School for Advanced Studies (SISSA), Via Bonomea 265, 34136 Trieste, Italy, Tel. 040.3787704, Fax 040.3787702, E-mail: cher@sissa.it
(2) Department of Neuroscience and Brain Technologies, The Italian Institute of Technology, Genova, Italy

Neuroligins (NLs) are postsynaptic cell adhesion molecules that interact with presynaptic neurexins (Nrxs) to ensure a correct cross talk between post and presynaptic specializations. One mutation causing the R451C substitution within the highly conserved region of the extracellular domain of Nlgn3 has been found in a few families with children affected by Autism Spectrum Disorders (ASD). Although rare, these alterations point to the synapse as a cause of Autism.
In relation to the specific aims of our project, we first used transgenic mice carrying the human R451C mutation of Nlgn3 to determine whether, early in postnatal life, GABAergic signaling is affected. Whole cell recordings from CA3 principal neurons in hippocampal slices from newborn animals revealed an enhanced frequency of Giant Depolarizing Potentials, a hallmark of developmental networks, in NL3R451C KI mice but not in control littermates. This effect was probably dependent on an increased GABAergic drive to principal cells as demonstrated by the enhanced frequency of miniature GABAA- but not AMPA-mediated postsynaptic currents. Changes in frequency were associated with an acceleration of miniatures decay possibly of postsynaptic origin. Therefore, it is reasonable to assume that alterations in the excitatory/inhibitory balance, crucial for the early refinement of neuronal circuits, accounts for the behavioral deficits observed in ASDs patients.
Preliminary experiments from regular-spiking neurons (in layer IV) in cortical slices from juvenile animals, revealed a statistically significant reduction in amplitude of GABAA-mediated inhibitory postsynaptic currents in NL3R451C KI but not control mice. No changes in the excitatory/inhibitory balance were observed in principal cells upon stimulation of thalamic inputs, which form direct excitatory synapses onto spiny neurons and parvalbumin positive interneurons, suggesting that NL3 control synaptic properties in a region- and circuit-specific manner.
NL3 localize at excitatory and inhibitory synapses where it interacts with post-synaptic components via various conserved motifs. To unveil the functional properties of these domains, in pull down experiments, several NL3 deletion mutants were generated and tested for their ability to interact with PSD95 and gephyrin. Preliminary data show that PSD95 interacts with NL3 lacking the PDZ binding domain, suggesting that other regions within the cytoplasmic domain mediate PSD95 recruitment.
To understand the functional interaction of GABA-A receptors (Rs) with the post synaptic scaffold gephyrin at GABAergic NL3R451C KI synapses we performed single particle tracking, an approach that allows visualizing the trajectories of individual receptors diffusing on the neuronal surface. Intriguingly, we observed that GABA-A Rs are significantly less immobile in NL3R451C KI respect to control litter mates, indicating a crucial role for NL3R451C mutation in stabilizing GABAARs at GABAergic synapses.

ABSTRACT N. 151

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**SICCA FEDERICO**	
Telethon grant N.	**GGP11188**	
Total budget €	**384.300**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

ROLE OF ASTROCYTIC INWARDLY-RECTIFYING K+ CHANNELS IN THE PATHOGENESIS OF AUTISM SPECTRUM DISORDERS WITH SUSCEPTIBILITY TO SEIZURES (AUTISM-EPILEPSY PHENOTYPE)

Ambrosini Elena (2), Pessia Mauro (3), Apicella Fabio (1), Baldini Sara (1), Brignone Maria Stefania (2), Conti Valerio (4), D'Adamo Maria Cristina (3), Guerrini Renzo (4,1), Lanciotti Angela (2), Marchese Maria (1), Moro Francesca (1), Santorelli Filippo Maria (1), Tancredi Raffaella (1), Valvo Giulia (1), Sicca Federico (1)

(1) Epilepsy, Neurophysiology and Neurogenetics Clinical and Research Unit, IRCCS Stella Maris Foundation, Viale dei giacinti 2, 56128 Calambrone (Pisa), Italy Tel. +39.050886234, Fax +39.050886247, E-mail: fsicca@inpe.unipi.it
(2) Section of inflammatory and demyelinating diseases of the central nervous system, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Roma
(3) Department of Internal Medicine, Section of Human Physiology, University of Perugia, Perugia
(4) Pediatric Neurology Unit, University Hospital A. Meyer, Florence

Background: The association of autism spectrum disorders (ASD) and epilepsy defines a condition termed Autism-Epilepsy Phenotype (AEP). This frequent comorbidity suggests shared pathogenetic mechanisms that could reside in glial inwardly-rectifying potassium channels (Kir) dysfunction. These channels are essential for neuronal and synaptic functioning, by regulating the astrocyte-mediated buffering of K+ in the brain. We have recently detected gain-of-function mutations of KCNJ10 (Kir4.1) in children with AEP, suggesting that the impairment of K+ buffering could contribute to both seizures and core features of ASD.
Objectives: In order to assess whether defects of Kir channels result in AEP, we are screening 150 patients with AEP for variants in KCNJ10 (Kir4.1), KCNJ16 (Kir5.1), and KCNJ2 (Kir2.1) genes. We are also characterizing them clinically to perform sharpened genotype-phenotype correlation. The functional effects of mutations on channel activity, protein trafficking, and astrocyte function, will be assessed in vitro, and in vivo on a knock-in KCNJ10 mouse model.
Methods: We are characterizing our sample clinically (seizures, EEG, behavior, cognition, and anthropometric data) and genetically (by direct Sanger sequencing of KCNJ10, KCNJ16, and KCNJ2). The functional effects of mutations are under investigation by using Xenopus laevis oocytes, cultured rat and mouse primary astrocytes, human astrocytoma/glioma cell lines, and by means of computational modeling in silico. A knock-in KCNJ10 mouse model (R18Q under construction) will be used to investigate disease mechanisms in vivo and in vitro, and for pharmacologic studies.
Results: We have so far screened 128 children and detected 4 missense KCNJ10 variants (R18Q, V84M, R348H, R271C) in a total of 14 individuals, and one change (K346T) in KCNJ2 in two sibs. We have also found that infantile spasms with good prognosis are the main type of seizures in patients harbouring mutations. The functional study of the K346T mutation showed that mutant Kir2.1 channels displayed increased current amplitude when expressed in Xenopus laevis oocytes, without differences in single channel conductance as compared to wild-type. In astrocytoma cells, the mutation enhanced surface expression and stabilization of channels at plasmamembranes. As seen for Kir4.1 mutations, it seems that also the K346T variant in Kir2.1 produces gain-of-function defects.
Conclusions: Preliminary data reinforce the role of astrocyte dysfunction in the pathogenesis of AEP. Assess the relative frequency of Kir channels defects in AEP, and their molecular consequences, will provide insight into the pathophysiology of both ASD and seizures, and will shed further light on the mechanisms by which astrocytes regulate neuronal excitability and synaptic function. This would improve diagnosis, and foster more rationalized therapeutic strategies.

ABSTRACT N. 152

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CHINI BICE**	
Telethon grant N.	**GGP12207**	
Total budget €	**136.300**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

OXYTOCIN ANALOGS IN PRADER-WILLI SYNDROME: NEW TOOLS TO INVESTIGATE AND TREAT SOCIAL AND COGNITIVE AUTISTIC-LIKE SYMPTOMS

Busnelli Marta (1,2), Leonzino Marianna (1,2), Ceresini Ilaria (1), Chini Bice (1)

(1) CNR Institute of Neuroscience, Via Vanvitelli 32, Milan, Italy, +39.02 5031695, +39.027490574, E-mail: b.chini@in.cnr.it
(2) Dept. Pharmacology, University of Milan, Milan, Italy

Background: Prader-Willi syndrome is a rare genetic disorder which affects one child in 25,000. Children born with this syndrome have severe feeding problems and a range of complex neurological and developmental disorders which continue into adult life. At present, treatment with psychotropic medications such as antidepressants, antipsychotics and appetite suppressants have shown limited effectiveness in controlling food-intake and there is no specific pharmacologic treatment for PWS behavioral disturbances.
The neuropeptide oxytocin (OXT) plays a central role in PWS pathogenesis: PWS individuals have a reduction in the number of OXT neurons in the hypohalamus and a deficit in circulating OXT. Because OXT regulates both feeding and social behavior, it represents a new promising approach for PWS, in which severe feeding disorders are associated to behavioral and social disturbances akin to autism. Pilot clinical trials have shown positive effects of intranasal OXT in autism, schizophrenia and social anxiety disorders; in a preliminary clinical trial, intranasal OXT has also been found to improve the ability of PWS individuals to read social cues and to facilitate their socialization PWS (Tauber et al Orphanet J Rare Dis 2011). However, OXT treatment is hampered by lack of receptor and coupling selectivity and the development of more selective and potent OXT analogues is needed to 1. understand the plethora of effects mediated by the natural peptide in the brain and 2. develop selective ligands effective on specific symptoms or groups of symptoms.
Aims: Our project is aimed at the development of new therapeutic tools to treat individuals suffering from PWS. These tools will target specific OXT-mediated effects identified by characterizing differentiation, synaptogenesis and synaptic plasticity in neuronal cultures obtained from oxytocin receptor (Oxtr) and Magel2 KO mice, two animal models with an impaired function of the OXT system. Notably, in the Magel2 KO mouse, a single administration of OXT at birth rescued the poor, life-threatening, feeding behavior of the pups (Schaller F et al, Hum Mol Genet 2010).
Experimental design: to unravel the cellular effects produced by the impairment of the OXT system in neuronal cells, we are developing neuronal cell cultures derived from Oxtr KO and Magel2 KO mice and we are characterizing the neuro-developmental processes modulated by OXT during synaptogenesis. Furthermore, as we have previously detected a GABA/glutamate imbalance in hippocampal cultures from Oxtr KO mice (Sala et al., Biol Psy, 2011) we are studying the role of OXT in modulating receptors, channels and transporters involved in GABA/glutamate mediated-effects. To identify new pharmacological tools targeting neuronal OXTRs, we are screening "functional-selective ligands", (Urban et al., J Pharmacol Exp Ther, 2007) and "bivalent ligands" (Messer, Curr Pharm Des, 2004), two class of drugs with still poorly characterized pharmacological properties.
Anticipated output: We expect to identify selective and highly potent analogues targeting brain OXTR to treat or ameliorate specific symptoms or groups of symptoms in PWS. Furthermore, these analogs will contribute to our understanding of the role of OXT in the neuropathology of conditions characterized by impaired OXT functioning, such as PWS and autism spectrum disorders.

ABSTRACT N. 153

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**SALA CARLO**	
Telethon grant N.	**GGP11095**	
Total budget €	**429.000**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2012**

IDENTIFICATION OF NEURONAL ALTERATIONS UNDERLYING SHANK3 MUTATIONS AND THEIR RESCUE BY GENETIC/PHARMACOLOGICAL THERAPIES IN ANIMAL MODELS AND PATIENTS' DERIVED IPS CELLS

Sala Carlo (1), Verpelli Chiara (1), Vicidomini Cinzia (1), Currelli Sebastiano (2), Broccoli Vania (3), Giustetto Maurizio (4), Dityatev Alexander (2)

(1) CNR Istituto di Neuroscienze, Via Vanvitelli 32, 20129 Milano, Tel.0250317096, Fax 027490574, email c.sala@in.cnr.it
(2) Dipartimento di Neuroscienze Fondazione Istituto Italiano di Tecnologia, Genova
(3) Division of Neuroscience, Fondazione Centro San Raffaele del Monte Tabor, Milano
(4) Dipartimento di Anatomia, Farmacologia e Medicina Legale, Università di

Torino e Istituto Nazionale di Neuroscienze, Torino

The 22q13 deletion syndrome is a genetic disease orphan of cure which cause a sever form of mental retardation and autism. The syndrome is also characterized by neonatal hypotonia, global developmental delay, absent to severely delayed speech, and minor dysmorphic features. The increasing number of patients being reported supports the hypothesis that this syndrome may be a common source of mental retardation. However, this genetic condition remains under-diagnosed due to failure to detect the 22q13 deletion in routine chromosome analysis and to recognize the phenotype on clinical examination. Lack of one copy of the SHANK3/PROSAP2 gene, encoding a structural protein located in the synapses of the human nervous system and involved in dendritic spines formation, is very likely the cause of the major neurological features associated with the deletion 22q13 syndrome. It has also been clearly demonstrate that mutation of SHANK3/PROSAP2 is associated to mental retardation and autism. The project aims to characterize the role of SHANK3 in the correct functioning of brain synapses. We applyed complementary techniques using also animal models and to be closer to the human pathology we established and differentiate to neurons human pluripotent cells from patients affected by the 22q13 deletion syndrome and age-matched healthy individuals in order to further clarify the neurons alterations underlying the syndrome. We are using these experimental models to test pharmacological and genetic rescue of the identified alterations.

ABSTRACT N. 154

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CATANIA MARIA VINCENZA**	
Telethon grant N.	**GGP07264**	
Total budget €	**279.000**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2007**

INVOLVEMENT OF GROUP-I METABOTROPIC GLUTAMATE RECEPTORS IN THE PATHOPHYSIOLOGY OF FRAGILE X SYNDROME

Bonaccorso Carmela Maria (1,2), D’Antoni Simona (1), Spatuzza Michela (1), Molinaro Gemma (3), Aloisi Elisabetta (1,4,5), Musumeci Sebastiano (2), Battaglia Giuseppe (3), Nicoletti Ferdinando (3,6), Davidovic Laetitia (7), Bardoni Barbara (7), Catania Maria Vincenza (1,2)

(1) ISN-CNR, Catania, Italy; - ISN-CNR, via Gaifami 18 95126 Catania, Italy Tel.+39/095-7338134 E-mail: m.catania@isn.cnr.it
(2) IRCCS Oasi Maria SS, Troina (EN), Italy
(3) Dept. of Neurosci., Neuromed, Pozzilli (IS), Italy
(4) PhD program in Neuropharmacology, University of Catania, Catania, Italy
(5) Institut National de la Santé et de la Recherche Médicale (INSERM), U862, Neurocentre Magendie, Univ. Bordeaux II, Bordeaux Cedex, France
(6) Dept. of Pharmacol., “La Sapienza”, Rome, Italy
(7) CNRS UMR 6097, Univ. de Nice Sophia-Antipolis, Nice, France

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability. It is caused by the absence of Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in the regulation of target mRNA translation and transport. FMRP is a component of mRNA ribonucleoprotein complexes and it can directly interact with several proteins, including other RNA binding proteins such as the Fragile X related proteins FXR1P and FXR2P. The functional significance of this interaction is presently unknown.
The involvement of group I mGlu receptors, namely mGlu5, in the pathophysiology of FXS is supported by several studies, in particular: 1) mGlu1/5 receptor activation triggers a rapid translation of dendritic mRNAs at synapses, including the FMRP mRNA; 2) protein synthesis-dependent, mGlu1/5 receptors-mediated forms of synaptic plasticity are increased in Fmr1 Knock Out (KO) mouse; 3) a reduced number of mGlu5 receptors are linked to the constitutive Homer proteins in Fmr1 KO mice; 4) mGlu5 receptor antagonists rescue the pathological phenotype in animal models of FXS. mGlu5 receptors blockade is now considered a valuable pharmacological strategy in FXS; however, the mechanisms underlying the altered mGlu5 receptor-mediated responses in FXS are not clear.
We studied and compared: 1) mGlu5 receptor expression, regional distribution, surface expression, dendrite/axon distribution and agonist induced internalization; 2) mGlu1/5 receptor-coupled polyphosphoinositide (PPI) hydrolysis; 3) glutamate release in freely moving mice; 4) protein expression profile in cortical synaptosomes; 5) the modulation of FMRP, FXR1P and FXR2P interaction by the activation of group I mGlu receptors, in wild type (WT) and Fmr1 KO mice.
We found that: a) mGlu5 receptors are more expressed in hippocampal synaptosomes of juvenile, but not adult Fmr1 KO mice; b) mGlu5 receptors are more expressed on the cell surface and do not undergo agonist-induced internalization in Fmr1 KO neurons. Furthermore, mGlu5 receptors are detectable in the axons in Fmr1 KO, but not in WT neurons; c) mGlu5 receptor-stimulated PPI hydrolysis is higher in young Fmr1 KO than WT mice, whilst is unchanged in the adult; d) the evoked glutamate release is strongly reduced in Fmr1 KO mice; e) differences between WT and Fmr1 KO mice are detectable in the expression profile of synaptic proteins prepared from young, but not adult animals; f) mGlu5 receptor activation reduces the interaction between FMRP/FXR2P in WT and FXR1P/FXR2P in Fmr1 KO mice.
Our results indicate that mGlu5 receptors are dysregulated when FMRP is absent and that the effects of lacking FMRP on mGlu5 expression and signalling are evident during development. Our data are also consistent with a potential involvement of the pre-synaptic compartment in the FXS phenotype. Furthermore, we have disclosed a novel function of mGlu5 receptors as modulators of the interaction between FMRP and its paralog proteins FXR1P and FXR2P.

ABSTRACT N. 155

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**NERI GIOVANNI**	
Telethon grant N.	**GGP10150**	
Total budget €	**253.500**	
Centres: **2**	*Duration (yrs):* **2**	*Starting year:* **2010**

MECHANISMS OF REACTIVATION OF THE FMR1 GENE AND ANALYSIS OF PATHWAYS INVOLVED IN THE PATHOGENESIS OF FRAGILE X SYNDROME: TOWARDS A DRUG-BASED THERAPY

Neri Giovanni (1), Bagni Claudia (2), Tabolacci Elisabetta (1), Zalfa Francesca (2), Chiurazzi Pietro (1)

(1) Istituto di Genetica Medica, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168 Roma, Italia, Tel. 06.3054449; Fax 06.30157223; E-mail: gneri@rm.unicatt.it
(2) Dipartimento di Biologia Sperimentale e Scienze Biochimiche, Facoltà di Medicina e Chirurgia, Università di Roma Tor Vergata, Roma, Italia

Fragile X syndrome (FXS) is the most common cause of inherited mental retardation and is due to loss-of-function mutations of FMR1, an X-linked gene containing a CGG repeat sequence in its promoter region. Expansion of this sequence beyond 200 repeats (full mutation) induces epigenetic changes that cause the transcriptional inactivation of FMR1 and absence of the corresponding protein FMRP. The Coordinator’s group has shown that FMR1 can be reactivated by treating FXS cells with the demethylating agent 5-azadeoxycytidine. We proposed to: 1) screen dozens of potentially reactivating compounds, in collaboration with the Broad Institute of Harvard and MIT and the company Neuropharm (UK); 2) characterize the epigenetic changes, critical for transcription, in FXS patients and in rare unaffected males, carrying an unmethylated full mutation (UFM) that is transcriptionally active; 3) analyze the epigenetic status of induced Pluripotent Stem (iPS) cells obtained from fibroblasts of FXS males, control individuals and UFM carriers. We can summarize our achievements as follows: 1) 20 compounds with potentially reactivating activity provided by Dr. Haggarty of the Broad Institute (Harvard & MIT) were tested on our model system i.e. FXS lymphoblastoid cells. Most of the compounds did not show any reactivating activity. We also expanded out original treatments with 5-azadC and followed up the reactivation for up to 4 weeks after treatment had been suspended and observed an unexpected increase in FMR1 transcription peaking some days after treatment and reaching transcription levels comparable to normal individuals. 2) we extended the analysis of the epigenetic modifications at the FMR1 locus in control, FXS and UFM individuals. The investigation included a recently described regulatory region, upstream of the gene’s promoter, and focused on the CTCF binding and its role in regulating transcription of FMR1 as well as antisense FMR1. 3) the collaboration with Dr. Guoping Fan (UCLA) was discontinued and Dr. Pirozzi moved to Leuven (VIB) where the Partner’s group has another laboratory in order to establish iPS cells there.
The Partner’s group has been interested since several years in the cellular and molecular events underlying spine dysmorphogenesis observed in FXS patients using a mouse model to study the syndrome (Fmr1 KO). Partner 1 has investigated, employing a microarray-based approach in collaboration with Dr. Victor Ambros (Umass, USA), the dysregulation of a subset of synaptic miRNAs in Fmr1 knockout mice. The next step was to identify some messenger

RNAs, targeted by those differentially expressed microRNAs, and further characterize them according to the effect they may have on spine morphology.

ABSTRACT N. 156

Telethon Research Projects - Neurological Diseases		
Principal Investigator		**CALEO MATTEO**
Telethon grant N.		**GGP11116**
Total budget €		**348.600**
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

ROLE OF OLIGOPHRENIN-1 IN CIRCUIT FORMATION AND FUNCTION IN A MOUSE MODEL OF X-LINKED MENTAL RETARDATION

Allegra Manuela (1,2), Spalletti Cristina (1,3), Redolfi Nelly (4), Murru Luca (5), Lodovichi Claudia (4,6), Passafaro Maria (5,7), Caleo Matteo (1)

(1) CNR Neuroscience Institute Via G. Moruzzi 1, 56124 Pisa, Italy, Tel. +39.050.3153195, Fax +39.050.3153220, E-mail: caleo@in.cnr.it
(2) Scuola Normale Superiore, Pisa
(3) Scuola Superiore S. Anna, Pisa
(4) Venetian Institute of Molecular Medicine (VIMM), Padua
(5) CNR Neuroscience Institute, Milan
(6) CNR Neuroscience Institute, Padua
(7) Dulbecco Telethon Institute (DTI), Milan

Intellectual disability (ID) is a complex disease of the central nervous system (CNS) whose etiopathogenesis is still poorly understood. The genetic contribution to the etiology of ID is well established and among the genetic conditions, the most frequent are the X-linked intellectual disability (XLID) forms caused by single gene mutations on chromosome X.
Among the X-Linked intellectual disability (XLID) genes, Oligophrenin-1 (OPHN1) encodes a synaptic RhoGTPase-activating protein that regulates neuronal morphology, i.e. shape of dendritic spines and outgrowth of axons in the brain. The involvement of OPHN1 in XLID was established by the identification of mutations within the gene in patients with XLID. More recent studies reported the presence of OPHN1 mutations in families with mental retardation associated with epilepsy and/or cerebellar hypoplasia. Oligophrenin-1 gene is expressed in brain areas that are characterized by high synaptic plasticity, in particular, the olfactory bulb and the hippocampus. At the cellular level, OPHN1 is expressed in both glial and neuronal cells where it colocalizes with actin, notably at the tip of growing neurites. The loss of function of OPHN1 is thought to cause abnormalities in neuronal morphology and wiring. These steps are essential for normal function of the CNS. Indeed distortions in neural circuit formation are associated to altered information processing, which is likely to be responsible of the cognitive impairment present in ID.
To investigate a role for OPHN1 in formation and function of neural circuits, we are analyzing the hippocampus and olfactory bulb of OPHN1 knock-out (KO) mice, an animal model of the disease. The olfactory bulb and hippocampus are particularly suited for these studies, as granule cells in the bulb and dentate gyrus constantly regenerate and undergo a series of well characterized morphological and functional changes to become fully mature neurons and integrate in the existing circuits. Accordingly, we are comparing neurogenesis and maturation of newborn neurons in wt and OPHN1 KO mice. Second, we are analyzing GABAergic circuits in the KO animals, as there is increasing evidence for alterations in inhibition in autism and ID. In particular, preliminary experiments in hippocampal neurons revealed a significant decrease in the peak amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in neurons lacking OPHN1. Interestingly, frequency of these events was unaltered after silencing OPHN1. These data suggest a role of OPHN1 in inhibitory synaptic transmission in hippocampal neurons. Ongoing anatomical studies are addressing the density of inhibitory innervation in the hippocampus and olfactory bulb of wt and OPHN1 KO mice.

ABSTRACT N. 157

Telethon Research Projects - Neurological Diseases		
Principal Investigator		**DE CURTIS IVAN**
Telethon grant N.		**GGP12126**
Total budget €		**284.800**
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

ROLE OF RHO FAMILY GTPases DURING NEURONAL DEVELOPMENT

Vaghi Valentina (1), Pennucci Roberta (1), Talpo Francesca (2), Corbetta Sara (1), Totaro Antonio (1), Montinaro Valentina (1), Barone Cinzia (1), Croci Laura (3), Spaiardi Paolo (2), D'Adamo Patrizia (3, 4), Bachi Angela (3), Consalez G. Giacomo (3), Biella Gerardo (2), de Curtis Ivan (1)

(1) Università Vita-Salute San Raffaele e Istituto Scientifico San Raffaele, via Olgettina 58, 20132 Milano, Tel. 02.26434828; Fax 02.26434844; decurtis.ivan@hsr.it
(2) Dipartimento di Scienze Fisiologiche e Farmacologiche, Università di Pavia
(3) Istituto Scientifico San Raffaele, Milano
(4) Dulbecco Telethon Institute presso Istituto Scientifico San Raffaele, Milano

The goals of our project are the characterization of the phenotypes induced by the genetic deletion of neuronal Rac1 and Rac3 GTPases in knockout (KO) mice models, and the analysis of the function of Rac effector complexes implicated in Mental Retardation during late neuronal development. During the first year, the project developed as follows:
Aim 1 - Analysis of the Mechanisms underlying prominent Defects in double KO (dKO) Brain. (a) We found that neither precursor proliferation, nor cell death and defects in migration account for the loss of mossy cells (MCs) in the hilus of dKO mice. (b) By the analysis of Rac's expression by in situ hybridization and synapsin-I-Cre expression we have identified GABAergic interneurons as a likely target of dKO. Accordingly, a striking defect in Lhx6-positive GABAergic interneurons in the cortex and hippocampus of dKO mice is observed, largely due to loss of parvalbumin (PV)-positive interneurons. Rac depletion also influences the maturation of interneurons that reach their destination, with reduction of inhibitory synapses in hippocampal and cortical pyramidal cells. The decreased number of migrating interneurons and their altered morphology indicate a role of Rac1 and Rac3 in regulating the motility of these cells, thus interfering with their final localization. These defects are likely contributing to the epileptic phenotype of dKO mice. Interestingly, mice with conditional KO of Rac1 in neurons show milder reduction of PV-positive interneurons, and become epileptic at adulthood. (c) Electrophysiological analysis of hippocampal circuits performed on brain sections shows higher sensitivity of dKO brain to induction of epileptic discharges by low doses of 4-aminopyridine compared to controls. While electrophysiological passive and active properties of pyramidal neurons including membrane capacity, resting potential, and spike amplitude and duration were normal, these cells showed reduced spontaneous inhibitory currents.
Aim 2 - Comparison between Rac1N and Rac3KO mice. Behavioral analysis of WT and Rac1N mice has shown that Rac1N mice show defects similar, although much more evident than those already described in Rac3-KO (Corbetta et al, 2008). Potential differences in other aspects of memory, fear, or anxiety are under investigation.
Aim 3 - Functional Interactions between Rac's and GIT Complexes in Developing Neurons. We have used Mass Spectrometry to identify a composite pattern of GIT/PIX/PAK complexes in the brain, which includes several isoforms of the betaPIX, alphaPIX, and PAK3 proteins, the last two being affected in Mental Retardation. No differences in the composition of these complexes between WT and mutant brains are observed. In a parallel study on hippocampal cultures we have shown strong inhibition of dendritic branching upon silencing of either alphaPIX or GIT2, suggesting that alphaPIX/GIT2 complexes are important for dendritic maturation, a pre-requisite for normal dendritic spine development.

ABSTRACT N. 158

Telethon Research Projects - Neurological Diseases		
Principal Investigator		**BROCCOLI VANIA**
Telethon grant N.		**GGP11110**
Total budget €		**349.700**
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2011**

MOLECULAR BASES AND IN VITRO MODELING OF CDKL5-DEPENDENT INFANTILE NEUROLOGICAL DISORDERS

Ricciardi Sara (1), Ungaro Federica (1), Hambrock Melanie (2), Rademacher Nils (2), Sessa Alessandro (1), Kalscheuer Vera M. (2), Broccoli Vania (1)

(1) Istituto Scientifico San Raffaele, Via Olgettina 58, 20132 Milan. Tel. 02.26434616. E-mail: broccoli.vania@hsr.it
(2) Max Planck Institute for Molecular Genetics, Department of Human Molecular Genetics, Berlin, Germany

Mutations of the cyclin-dependent kinase-like 5 (CDKL5) and netrin-G1 (NTNG1) genes cause a severe neurodevelopmental disorder with clinical features that are closely related to Rett syndrome, including intellectual disability, early-onset intractable epilepsy and autism. We report here that CDKL5 is localized at excitatory synapses and contributes to correct dendritic spine structure and synapse activity. To exert this role, CDKL5 binds and phosphorylates the cell adhesion molecule NGL-1. This phosphorylation event ensures a stable association between NGL-1 and PSD95. Accordingly, phospho-mutant NGL-1 is unable to induce synaptic contacts whereas its phospho-mimetic form binds PSD95 more efficiently and partially rescues the CDKL5-specific spine defects. Interestingly, similarly to rodent neurons, iPSC-derived neurons from patients with CDKL5 mutations exhibit aberrant dendritic spines, thus suggesting a common function of CDKL5 in mice and humans.

ABSTRACT N. 159

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**CIANI ELISABETTA**	
Telethon grant N.	**GGP11147**	
Total budget €	**458.700**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2011**

CDKL5, A NOVEL GENE INVOLVED IN A VARIANT OF RETT'S SYNDROME, PLAYS A KEY ROLE IN THE REGULATION OF NEURONAL PRECURSOR CELL PROLIFERATION, DEATH AND DIFFERENTIATION

Trazzi Stefania (1), Fuchs Claudia (1), Emanuele Valli (2), Amendola Elena (3), Giustetto Maurizio (4), Pizzorusso Tommaso (5), Calza' Laura (6), Bartesaghi Renata (1), Gross Cornelius (3), Perini Giovanni (2), Ciani Elisabetta (1)

(1) Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Tel. 051.2091726, Fax 051.2091737, E-mail: elisabetta.ciani@unibo.it
(2) Dipartimento di Farmacia e Biotecnologie, Università di Bologna
(3) European Molecular Biology Laboratory, EMBL, Monterotondo (Roma)
(4) Dipartimento di Anatomia, Farmacologia e Medicina Legale, Università di Torino
(5) Istituto di Neuroscienze del CNR, Pisa
(6) Dipartimento di Scienze Mediche Veterinarie, Università di Bologna

Rett's Syndrome (RTT) is a progressive neurodevelopmental disorder characterized by precocious arrest of neurological development and cognitive decline. Recently, mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5) gene have been detected in RTT patients characterized by early-onset seizures. Despite the clear involvement of CDKL5 mutations in this variant of RTT, the function/s of this protein during brain development and the molecular mechanisms involved in its regulation remain to be elucidated. In the current study we have used an in vitro and an in vivo approach in order to obtain novel evidence on the role of CDKL5 in fundamental processes of brain development.
Using human neuroblastoma cell lines as a neuronal model system, we found that inhibition of CDKL5 expression increased cell proliferation and reduced neuronal differentiation. Conversely, an increase in CDKL5 expression caused cell cycle arrest and favoured differentiation. This evidence indicates that CDKL5 acts as anti-proliferative and pro-differentiative gene in neuronal cells. Interestingly, CDKL5 expression was inhibited by MYCN, a transcription factor that promotes cell proliferation during brain development. We found that MYCN acts as a direct repressor of the CDKL5 promoter, suggesting a functional axis between MYCN and CDKL5. Inhibition of the CDKL5 promoter by MYCN was confirmed in cerebellar granule cells, indicating a MYCN-dependent inhibition of CDKL5 expression in different types of neural precursors. In order to mimic the human pathology, we have generated a constitutive Cdkl5 knockout mouse by deletion of exon 4 of the Cdkl5 gene. Western blot analysis of whole brain extracts and immunofluorescence of brain sections confirmed the absence of Cdkl5 protein in hemizygous male and homozygous female knockout mice and intermediate levels in heterozgous females. Cdkl5 knockout mice are viable and can thus be exploited to study the effects of CDKL5 mutations in vivo. We found that Cdkl5 knockout mice showed a higher neuronal precursor proliferation rate both in the hippocampal dentate gyrus and subventricular zone with respect to wild type animals, confirming the anti-proliferative role of CDKL5 observed in vitro. Interestingly, these areas were also characterized by an increased rate of apoptotic cell death that caused a reduction in final neuron number in spite of the proliferation increase.
Overall our findings highlight a critical role of CDKL5 in the control of neuron proliferation, death and differentiation and lay the bases to a better understanding of the neurological symptoms during the early period of brain maturation in patients carrying mutations in the CDKL5 gene.

ABSTRACT N. 160

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**TONGIORGI ENRICO**	
Telethon grant N.	**GGP08258**	
Total budget €	**99.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

PHARMACOLOGICAL APPROACHES TO RESTORE BDNF LEVELS IN CELLULAR AND ANIMAL MODELS OF THE RETT SYNDROME

Bittolo Tamara, Baj Gabriele, Vaghi Valentina, Tongiorgi Enrico

Brain Centre for Neuroscience, Dipartimento di Scienze della Vita, Università di Trieste, Via Giorgieri, 5 - edif.Q, 34127 Trieste, Tel. 040.5588724, E-mail: tongi@units.it

The Rett syndrome (RTT) is the major cause of mental retardation in female children. This pathology is caused by mutations affecting the MECP2 gene located on chromosome X that in normal conditions regulates the expression of numerous genes. Brain Derived Neurotrophic Factor (BDNF) is expressed at lower levels in mouse models of RTT and two recent studies have shown that in these mice, it is possible to reverse the disease course by overexpressing BDNF. Using real-time PCR, we found different expression pattern of BDNF transcripts in age-matched normal and RTT samples of primary somatosensory and motor cortex depending on the mutation in MeCP2 gene. Furthermore, we determined the BDNF isoforms in forebrain cortex and hippocampus in MeCP2 null mice (Bird) and wt animals at different postnatal ages. Considering the complex technical problems involved in gene therapy, we decided to follow a pharmacological approach to treat the Rett syndrome. Hence, using a luciferase-based translational in vitro assay we carried out a systematic pharmacological analysis to determine which drug can be used to enhance BDNF protein levels. We found that a combination of serotonin and norepinephrine was the most effective stimulus for BDNF synthesis. Then, we tested MeCP2 KO mice, the effects of desipramine and mirtazapine, two antidepressants which induce combined enhancement of serotonine and norepinephrine. We found that upon two weeks of treatment the cortical thickness, which is 20% smaller in MeCP2 KO mice with respect to wild type, is partially rescued with desipramine and better with mirtazapine. Future clinical applications are possible, since the investigated compounds are already approved for human use.

ABSTRACT N. 161

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**RENIERI ALESSANDRA**	
Telethon grant N.	**GGP09117**	
Total budget €	**293.100**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2009**

CONGENITAL RETT SYNDROME: CELLULAR AND MOUSE MODELS FOR THE STUDY OF FOXG1 IMPACT ON FOREBRAIN NEUROGENESIS

Meloni Ilaria (1), Broccoli Vania (2), Livide Gabriella (1), Ricciardi Sara (2), Mari Francesca (1), Ariani Francesca (1), Renieri Alessandra (1)

(1) Medical Genetics, Department of Biotechnology, University of Siena, Phone +39.0577.233303, Fax +39.0577.233325, E-mail: renieri@unisi.it
(2) San Raffaele Scientific Institute, Dibit, Milano, Italy

Classic Rett syndrome (RTT) is due to mutations in the transcriptional regulator MECP2. Mutations in FOXG1 gene are responsible for the congenital RTT variant. FOXG1 encodes for a transcriptional repressor that plays an essential role in the development of telencephalon in embryonic mammalian forebrain. In particular, Foxg1 has been implicated in regulating cortical arealization and progenitor cells proliferation. Its expression persists in post-natal brain, suggesting an important role also in postmitotic neurons. The similar phenotypes resulting from mutations in the two genes suggested that they may interact or at least participate in common pathways. To verify this hypothesis we performed co-immunoprecipita-

tion experiments in HEK293T cells and primary neurons but we did not detect a direct interaction. It is however still possible that the two proteins might participate to common pathways though without direct interaction. Since they are both involved in transcriptional control, such pathways might involve the regulation of the expression of specific genes. Despite intense research efforts aimed at understanding FoxG1 function, very few target genes have been identified. Thus, to identify such targets and to verify whether genes common to MeCP2 network exist, we compared expression profiles in whole brain from Foxg1 +/- heterozygous mice and wild type littermates at postnatal day 30. The experiment allowed the identification of 55 genes with altered expression: 28 up-regulated and 27 down-regulated genes. A set of 15 genes have been selected for further validation based on their function.
Silencing of MECP2 in cultured mouse neurons allowed the identification of specific phenotypes consistent with the observations on autoptic brain. In order to determine whether similar changes could be observed in the absence of Foxg1 and to define its function at the cellular level, we manipulated Foxg1 expression in cultured neurons. Down-regulation of Foxg1 by shRNA resulted in a significant increase in dendritic branching and in the appearance of thin, immature spines; spines density was also increased. The over-expression of Foxg1 resulted in a reduction of dendritic branching; however, no effect on dendritic spines was observed, apart from a reduction of their length.
In 2007 it was demonstrated that it is possible to reprogram human fibroblasts into induced pluripotent stem (iPS) cells and to differentiate them into neurons. iPS technology thus offers the unprecedented opportunity to recapitulate normal and pathologic human tissue formation in vitro. Therefore, at the beginning of the project we planned to obtain iPS from fibroblasts of FOXG1-mutated patients and to differentiate them into neurons. After many attempts we could finally reprogram fibroblasts from one patient with a truncating FOXG1 mutation (p.S323fsX325). Clones characterization has been recently completed and we are now proceeding with neuronal differentiation.

ABSTRACT N. 162

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**PIZZORUSSO TOMMASO**	
Telethon grant N.	**GGP09196**	
Total budget €	**482.200**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2009**

IDENTIFICATION OF NEURONAL SUBSTRATES OF RETT SYNDROME AND VALIDATION OF THERAPIES IN PRECLINICAL MODELS: A GENOMIC, MORPHOFUNCTIONAL, AND BEHAVIOURAL ANALYSIS IN MOUSE MODELS AND PATIENTS

Pizzorusso Tommaso (1), Battini Roberta (4), Biagi Laura (4), Calcagno Eleonora (2), Casarano Manuela (4), Cea Valentina (3), Cioni Giovanni (4), Giustetto Maurizio (2), Guzzetta Andrea (4), Landi Silvia (5), Lonetti Giuseppina (1), Morello Noemi (2), Pini Giorgio (6), Putignano Elena (1), Ratto Gian Michele (5), Sala Carlo (3), Tosetti Michela (4)

Istituto di Neuroscienze del CNR, via Moruzzi 1, 56124, Pisa, Tel.+39.050.3153167, Fax +39.050.3153220, E-mail: pizzorusso@in.cnr.it
(2) Dept. of Neuroscience and Natl. Inst. of Neuroscience, Univ. of Torino
(3) Istituto di Neuroscienze del CNR, Milano
(4) Division of Child Neurology and Psychiatry, University of Pisa, Stella Maris Scientific Institute, Calmbrone, Pisa
(5) National Enterprise for Nanoscience and Nanotechnology, Istituto Nanoscienze, Consiglio Nazionale delle Ricerche, Pisa
(6) Tuscany Rett Center, Versilia Hospital, 55043 Lido di Camaiore, Lucca

Rett Syndrome is a postnatal progressive disorder with no efficient treatment that manifests mainly in girls during early childhood. The large majority of the RS patients carry a mutation of the MeCP2 gene.
We studied the role of MeCP2 in neuronal development in vitro and in vivo. Cortical neurons were infected with lentivirus expressing the shRNA for MeCP2 (shMeCP2) or shCTRL (control) at DIV3-4 and stained at DIV7 and DIV14. We then analyzed the axonal and dendrite development using Tau and MAP2 antibodies. We found that MeCP2 silencing reduces the neuronal development of both dendrites and axon and the number of dendritic spines as previously demonstrated. The same neurons were used for proteomic analysis using SILAC methodology for studying the differential protein expression. We are now proceeding for mass-spectrometry analysis of the labelled proteins.
The in vivo impact of MeCP2 mutation on the development and maturation of excitatory and inhibitory circuits was investigated. We report that Mecp2 deletion may induce significative alteration in the density and distribution of some but not all subtypes of GABAergic interneurons in the cerebral cortex. While birth and migration of somatostatin (SST)-positive interneurons seems normal, the laminar density of both parvalbumin- (PV) and calretinin- (CR) positive interneurons in MeCP2-KO mice is abnormally increased at an early postnatal developmental stage when animals are still asymptomatic. Moreover, both the differentiation and morphological organization of the CR+ interneuron subpopulation is affected in these mutants. Interestingly, we found that 10 months old heterozygous female (MeCP2+/-) mice, a model closely mimicking many aspects of RTT symptoms, recapitulate most of the interneurons alterations shown by male null-mice. We propose that an abnormal pattern of excitatory axonal wiring in the cortex is likely to produce alteration in the balance between excitation and inhibition in the synaptic circuits of MeCP2 mutant animals.
Dendritic spines are also affected in MeCP2 mutants. To monitor spine dynamics in MeCP2 mutants we used in vivo two-photon microscopy on MeCP2-GFP mice. We found that MeCP2 mutation results in impaired filopodia outgrowth and plasticity suggesting that the processes involved in regulation of filopodial protrusion are at basis of the spine defects present in MeCP2 mutants.
To assess circuit alterations in Rett Syndrome patients, we analyzed 13 patients with MeCP2 mutation (range of age 2-21 yrs). All patients underwent to a regular and periodic clinical follow up with EEG and a special assessment for respiratory features. Single-pulse TMS showed a reduced time motor conduction. MRI examination, with functional and anatomical profiles (Voxel-Based Morphometry, VBM; Arterial Spin Labelling, ASL; Diffusion Tensor Imaging, DTI), was performed in 10 patients of our sample and data analysis and matching with appropriate controls is still undergoing.

ABSTRACT N. 163

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**LANDSBERGER NICOLETTA**	
Telethon grant N.	**GGP10032**	
Total budget €	**398.600**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2010**

MECP2 PHOSPHORYLATION AND RELATED KINASES IN RETT SYNDROME AND EARLY INFANTILE EPILEPTIC ENCEPHALOPATHY 2

Bergo Anna (1), Strollo Marta (1), Kisltrup-Nielsen Charlotte (1), Soddu Silvia (2), Landsberger Nicoletta (1)

(1) Department of Theoretical and Applied Science, Lab. of the Genetic and Epigenetic Control of Gene Expression, University of Insubria, Busto Arsizio, Varese, Italy, Tel. 02.26436278, Fax 02.26434844, E-mail: landsberger.nicoletta@hsr.it
(2) Dipartimento di Oncologia Sperimentale, Istituto Tumore Regina Elena, Roma

Dramatic progress has been made in the last 12 years, since the discovery of MECP2 as the Rett (RTT)-causing gene, providing insight into the pathogenesis of the disease. Generally, MeCP2 is considered an ubiquitously expressed methyl-DNA binding protein that represses transcription by promoting chromatin compaction. However, recent studies have proposed that MeCP2 might also have activating functions. MeCP2 differential phosphorylation might render it a multifunctional protein. Indeed, even though transcriptional regulation is still considered the main function of MeCP2, other roles have been proposed. One report demonstrated an interaction of MeCP2 with the protein YB1 suggesting an involvement of the methyl-binding protein in alternative splicing, whereas our group has contributed proposing that RTT may be also given by hypofunctional protein synthesis in brain cells.
With this communication, we propose yet another function of this protein. We demonstrate that MeCP2 is a constituent of the centrosome. In fact, by immunofluorescence we have demonstrated that, in cultured cells, MeCP2, and, in particular, one of its specific phospho-isoforms, localizes in the centrosome. Importantly, the centrosome localization is observed also in primary hippocampal and cortical neurons and also by centrosomal fractionation experiments. The presence of MeCP2 in centrosomes and the validity of the used antibodies have been validated by shRNA MeCP2 knock-down or by using Mecp2-null neurons. Phenotypically, Mecp2-defective cells are characterized by a severely impaired capability to nucleate microtubules and cytoplasmic asters, defective cell-cycle progression and

show altered centrosome numbers. Eventually, we have initiated to assess the expression and the activity of several kinases involved in centrosome function (i.e. Aurora kinases and PLK1) in MeCP2-ablated cells. Interestingly, our preliminary results find clear phenotypic effects.
Even though existing results are contradictory, the centrosome seems not only to provide a structural hub for the microtubule array but appears as the major microtubule nucleation site and is involved in neuronal polarization, maturation and migration. Therefore, it is not surprising that several permanent or transient components of the centrosome have been associated with diverse neuropsychiatric diseases. Thus, we propose that the novel centrosomal function of MeCP2 might be of relevance for neuronal maturation and differentiation and, therefore, has to be considered in the pathogenesis of Rett syndrome and MECP2-related disorders. Accordingly, a Mecp2 knock-in mouse defective in the phospho-site specifically associated with MeCP2 association in the centrosome has recently been obtained by us and preliminary results suggest the presence of a RTT-like phenotype.

ABSTRACT N. 164

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**MELDOLESI JACOPO**	
Telethon grant N.	**GGP09066**	
Total budget €	**442.300**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2009**

EXPRESSION AND EFFECTS OF L1CAM AND ITS MISSENSE MUTATIONS IN PC12 CELLS AND NEURONS

Tagliavacca L., Mikulak J., Colombo F., Racchetti G., Meldolesi J., Valente P., Medrihan L., Bosco F., Baldelli P.

Istituto Scientifico San Raffaele, Via Olgettina 58, 20132 Milan

Our project is focused on the expression of a gene, that of the adhesion protein L1CAM, whose missense mutations are known to sustain a genetic disease the L1 syndrome or CRASH. This disease is quite variable in terms of severity and also of symptoms exhibited by the patients. The variability is such that for many years the disease was interpreted as a few different syndromes that were grouped together only when the pathogenesis was finally clarified.
The expression of L1CAM is governed by the transcription repressor REST/NRSF, the master factor of neural cell differentiation which is active also in mature neurons. At variance with non-neural cells, the levels of REST are very low in neurons and neural cells but can increase, although moderately, upon stimulation or injury, and this could induce decreased expression of its targets, including L1CAM. Our work was developed in two directions.
First we have studied REST and L1CAM in PC12 cells and found that REST governs not only the level but also the splicing of L1CAM, dependent of its target Nova2 (Mikulak et al., JNC 120: 699-709, 2012). This finding is important because the high REST spliced form is less effective than the low REST, full length form.
Subsequently we have studied four missense mutants of the surface L1CAM domain and found that each of them affects distinct functions of the protein.
In addition we have discovered a new function of L1CAM, a robust, dose dependent modulation of the TrkA receptor of NGF which is shared by two but not of the other two L1CAM mutants. This finding, which may be relevant for some of the brain lesions observed in CRASH patients, can account for the heterogeneity of the clinical pictures induced by the various mutants (Tagliavacca et al., JNC 2012 Sep 13. doi: 10.1111/jnc.12015).
In parallel, primary hippocampal cultures obtained from L1CAM KO mice, have been used as model of the disease. Multielectrodes array recordings showed a reduced spontaneous firing activity in KO neuronal networks. Patch-clamp recordings revealed that, with respect to wt neurons, L1CAM KO neurons are characterized by a more negative resting potential, higher input resistance and rheobase, lower voltage-threshold and action potential amplitude. In accord with these changes, we observed a reduced expression of voltage-gate Na+ channels (Nav) and Na+ current density. Moreover, confocal microscopy showed an altered localization of Nav at the axonal initial segment accompanied by a reduced axonal elongation in L1CAM KO neurons. This unexpected action of L1CAM deletion suggests a new role of the adhesion protein, affecting specifically the functional maturation of membrane excitability in developing hippocampal neurons.

ABSTRACT N. 165

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**SERINI GUIDO**	
Telethon grant N.	**GGP09175**	
Total budget €	**295.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

THE R-RAS/RIN2/RAB5 COMPLEX CONTROLS ENDOTHELIAL CELL ADHESION AND VASCULAR MORPHOGENESIS VIA ACTIVE INTEGRIN ENDOCYTOSIS AND RAC SIGNALING

Sandri Chiara (1,2), Caccavari Francesca (1,2), Valdembri Donatella (1,2), Camillo Chiara (1,2), VelTel.Stefan (3), Santambrogio Martina (1,2), Lanzetti Letizia (2,4), Bussolino Federico (2,5,6), Ivaska Johanna (3), Serini Guido (1,2,6)

(1) Laboratory of Cell Adhesion Dynamics, Institute for Cancer Research and Treatment (IRCC), Strada Provinciale 142 Km 3.95, 10060 Candiolo (TO), Italy, Tel. +39.011.9933508, Fax +39.011.9933524, E-mail: guido.serini@ircc.it
(2) Department of Oncological Sciences, University of Torino School of Medicine, 10060 Candiolo, Italy
(3) University of Turku Centre for Biotechnology and VTT Medical Biotechnology, 02044 Turku, Finland
(4) Laboratory of Membrane Trafficking, Institute for Cancer Research and Treatment (IRCC), Candiolo, Italy
(5) Laboratory of Vascular Oncology, Institute for Cancer Research and Treatment (IRCC), Candiolo, Italy
(6) Center for Complex Systems in Molecular Biology and Medicine - SysBioM, University of Torino, c/o Department of Animal and Human Biology (DBAU), Torino, Italy

During developmental angiogenesis, secreted class 3 semaphorins (SEMA3) [1] regulate blood vessel navigation by signaling through neuropilin [2] and plexin receptors that inhibit the R-Ras subfamily of small GTPases [3]. R-Ras is mainly expressed in vascular cells [4], where it induces adhesion to the extracellular matrix (ECM) through unknown mechanisms [5]. We identify the Ras and Rab5 interacting protein RIN2 as a key effector that in endothelial cells interacts with and mediates the pro-adhesive and -angiogenic activity of R-Ras [6,7]. Both R-Ras-GTP and RIN2 localize at nascent ECM adhesion sites associated with lamellipodia, where, upon binding, GTP-loaded R-Ras converts RIN2 from a Rab5 guanine nucleotide exchange factor (GEF) to an adaptor that first interacts with high affinity Rab5-GTP to promote the selective endocytosis of ligand-bound/active ß1 integrins and then causes the translocation of R-Ras on early endosomes. Here, the R-Ras/RIN2/Rab5 signaling module activates Rac1-dependent cell adhesion via TIAM1, a Rac GEF that localizes on early endosomes and is stimulated by the interaction with both Ras proteins and the vesicular lipid phosphatidylinositol 3 monophosphate. In conclusion, the ability of R-Ras-GTP to convert RIN2 from a GEF to an adaptor that preferentially binds Rab5-GTP allows triggering the endocytosis of ECM-bound/active ß1 integrins [8] and the ensuing funneling of R-Ras GTP towards early endosomes to elicit the pro-adhesive and TIAM1-mediated activation of Rac1.
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ABSTRACT N. 166

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**DI CUNTO FERDINANDO**	
Telethon grant N.	**GGP12095**	
Total budget €	**270.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

IDENTIFICATION OF THERAPEUTIC TARGETS IN PRIMARY MICROCEPHALY THROUGH THE ANALYSIS OF THE CIT-K/ASPM PATHWAY

Bianchi Federico (1), Gai Marta (1), Chang Yoon Jeung (2), El Assawy Nadia (3), Di Cunto Ferdinando (1)

(1) University of Torino, Molecular Biotechnology Centre, Via Nizza 52, 10126, Torino, Italy, Tel. +39.011.6706409, Fax +39.011.6706432, E-mail: ferdinando.dicunto@unito.it
(2) Max Planck Institute of Molecular Cell Biology and Genetics, MPI-CBG, Dresden, Germany
(3) University of Torino, Department of Neurology and Neurorehabilitation, IRCCS, Istituto Auxologico Italiano, Piancavallo (VB)

Genetic microcephaly comprises a heterogeneous group of disorders that share a small brain size and that can be associated with strongly invalidating symptoms, such as intellectual disability, epilepsy, ataxia and limb paralysis. Enormous progress has so far been achieved in the definition of the genetic basis of microcephalies. Nevertheless, it is still hard to envisage how this knowledge could be translated into new therapies. The study of the cellular and of the molecular mechanisms involving microcephaly genes is therefore extremely important, not only from a general scientific point of view but also because it has the potential to reveal molecular crossroads amenable to therapeutic intervention. Most of the identified microcephaly genes share a ubiquitous expression pattern and are involved in mitosis, but it is presently unknown why their inactivation leads to a specific decrease of the brain volume. Reduction of the symmetric cell divisions responsible for the expansion of the neuronal progenitors' pool during corticogenesis is one of the main proposed mechanisms. In addition, or in alternative, the same mutations may lead to other types of brain-specific cell proliferation abnormalities, ending with the apoptotic loss of neuronal precursors. Mice lacking Citron-kinase (CIT-K), which are characterized by microcephaly, ataxia and lethal epilepsy, are a very interesting model to study these problems. Indeed, first of all we have found that these mice may display both mechanisms of neuronal depletion. Moreover, we show that CIT-K may functionally interact with other microcephaly genes, such as ASPM. Finally, and perhaps most importantly, we provide a strong proof of concept that the CIT-K phenotype can be counteracted through 'drugable' mechanisms. In this project we aim to extend our current studies of the molecular, cellular and developmental events that involve CIT-K and its partners, with particular emphasis on ASPM.

ABSTRACT N. 167

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**PLEVANI PAOLO**	
Telethon grant N.	**GGP11003**	
Total budget €	**295.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE ROLE OF RNaseH2 IN THE PATHOGENESIS OF AICARDI-GOUTIÉRES SYNDROME

Pizzi Sara (1), Lazzaro Federico (1), Novarina Daniele (1), Delmastro Daria (1), Tamolli Giada (1), Sertic Sarah (1), Granata Magda (1), Panigada Davide (1), Burgers Peter (2), Kunkel Thomas (3), Muzi-Falconi Marco (1), Plevani Paolo (1)

(1) Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy
(2) Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO U.S.A.
(3) Laboratory of Structural Biology and Molecular Genetics, NIEHS, Research Triangle Park, NC, U.S.A.

Aicardi-Goutières syndrome (AGS) is a genetic encephalopathy whose clinical features mimic congenital viral infection and, for this reason, is frequently misdiagnosed. Mutations in six genes (AGS1-6) are responsible for the AGS forms. All these genes code for proteins acting as nucleases or nucleotide modifiers; ~65 % of the patients carry mutations in AGS2, AGS3, AGS4, the genes encoding the 3 subunits of human RNaseH2. RNases H are enzymes cleaving the RNA moiety in RNA:DNA hybrid molecules. Eukaryotic cells possess RNaseH1 and RNaseH2 activities that have partially overlapping substrate specificity. RNase H1 requires a tract of 4 ribonucleotides (rNMPs) to cleave, while RNaseH2 can incise a single rNMP within a DNA molecule. RNaseH1 is single polypeptide essential for mitochondrial DNA replication, while RNaseH2 is a 3 subunits complex whose function in replication and transcription are still poorly defined. To better understand the mechanisms linking RNaseH2 mutations to AGS pathogenesis we are combining genetic and molecular biology approaches in yeast and human cells. Using the yeast model system it has been found that the integrity of the genome is challenged during DNA replication by the incorporation of rNMPs. This misincorporation is generally prevented by the selectivity of DNA polymerases. Nonetheless, DNA polymerase epsilon, the enzyme involved in leading strand DNA synthesis, incorporates rNMPs with a surprisingly high frequency. We found that both RNaseH1 and RNaseH2 have roles in removing rNMPs from DNA, and failure to do so causes replication stress when DNA polymerases encounter rNMPs in the template strand at the next round of replication. We provided genetic and biochemical evidence that the ability of RNase H double-mutant cells to survive replication stress depends on the two post-replication (PRR) pathways. Finally, cells lacking RNaseH have a constitutively activated PRR and accumulate ubiquitylated PCNA (Lazzaro et al., 2012, Mol. Cell, 45, 99-110).
We discovered that simultaneous expression of the human RNaseH2 genes in yeast is able to complement the phenotypes associated with deletions of the corresponding yeast genes, making feasible the use of yeast cells as a tool to study how mutations in human genes found in AGS patients affect RNaseH2 function. We are also transfering the knowledge gained in yeast to further investigate the role of RNaseH on genome stability in human cells. By inducing stable depletions of RNaseH1 and RNaseH2, silenced human cells are analyzed for their hypersensitivity to replication stress inducing agents, cell cycle and proliferation defects. The level of ubiquitylated PCNA is also used as a readout of PRR activation. Altogether, our findings describe a new function for RNaseH and PRR in overcoming the obstacles represented by rNMPs incorporated during DNA replication.

ABSTRACT N. 168

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**SALLESE MICHELE**	
Telethon grant N.	**GGP12220**	
Total budget €	**357.700**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2013**

PURKINJE CELL DEGENERATION IN MARINESCO-SJOGREN SYNDROME: ROLE OF CELL STRESS, ALTERATIONS OF PROTEOSTASIS AND CALCIUM HOMEOSTASIS

Ruggiero Carmen (1), Grossi Mauro (1), Fragassi Giorgia (1), Capone Vanessa (1), Pozzoli Manuela (2,3), Bertani Ilaria (2,3), Restelli Elena (2,3), Zanardi Alan (2,3), Chiesa Roberto (2,3), Sallese Michele (1)

(1) Unit of Genomic Approaches to Membrane Traffic, Department of Cellular and Translational Pharmacology. Consorzio Mario Negri Sud, Via nazionale 8/a, Santa Maria Imbaro (Chieti), Tel. 0872.570421, Fax 0872.570412, E-mail: sallese@negrisud.it
(2) Dulbecco Telethon Institute, Milano
(3) Laboratory of Prion Neurobiology, Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Milano

The Marinesco-Sjogren syndrome (MSS) is a rare, early-onset, autosomal recessive genetic disease caused by mutations in the SIL1 gene. One of the main symptoms of MSS is ataxia due to degeneration of Purkinje cells (PC). To date there is no treatment for MSS, and the medical care of the patients is essentially symptomatic. SIL1 encodes an ADP exchange factor for GRP78, the master operator of endoplasmic reticulum (ER) functions. Eukaryotic cells express a second ADP exchange factor, ORP150, potentially able to compensate for the loss of SIL1 function. However, ORP150 overexpression seen in mouse models of MSS (woozy), apparently is not sufficient to prevent PC apoptosis, suggesting that induction of ORP150 could be sufficient to rescue for SIL1 loss only in certain tissues.
To elucidate the basis of PC degeneration we plan to investigate

SIL1/GRP78-dependent cell processes, including the activating of ER stress pathways (unfolded protein response, UPR), the protein folding/transport, the gating of the translocon, and ER-associated degradation in woozy mice and in MSS cells in which SIL1 expression is transiently or stably knocked down (KD).
Because in MSS patients different tissues are differently affected, we will use different cell lines, including HeLa (cervical cancer) and SH-SY5Y (neuroblastoma). Preliminary data show that HeLa and SH-SY5Y cells express comparable levels of SIL1while expression of ORP150 is higher in SH-SY5Y. This suggests that HeLa cells might be more sensitive to SIL1 loss. SIL1 KD induced ORP150 expression in both SH-SY5Y and HeLa cells. Interestingly, SIL1 KD activates the UPR in HeLa cells as monitored by PDI overexpression. We hypothesize that in this cells ORP150 overexpression is not sufficient to rescue the ADP exchange function on GRP78, inducing the UPR.
In addition, we have evidence that membrane trafficking is impaired in SIL1 KD HeLa cells. This indicates that SIL1 KD elicits a functional impairment that could affect the trafficking and distribution of ion channels or neurotransmitter receptors leading to PC dysfunction and degeneration.

ABSTRACT N. 169

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BANKS LAWRENCE**	
Telethon grant N.	**GGP10006**	
Total budget €	**194.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

THE ANGELMAN SYNDROME ASSOCIATED UBE3a UBIQUITIN LIGASE CONTROLS PROTEASOME FUNCTION

Tomaic Vjekoslav, Massimi Paola, Banks Lawrence

ICGEB, Padriciano 99, Trieste, Italy. Tel +39.040.3757328; E-mail: banks@icgeb.org

Angelman Syndrome is a neurological disorder characterised by severe developmental defects. In the majority of cases development of the Syndrome is associated with either loss or mutation of the maternal copy of the gene encoding for UBE3a. This protein is a ubiquitin ligase whose primary function is believed to be the regulation of protein expression levels. This occurs through its ability to conjugate ubiquitin to its substrate proteins, which in turn are degraded at the 26S proteasome. Previous studies had indicated that loss of enzymatic activity phenocopied loss of the entire gene, suggesting that the enzymatic activity was a central aspect of its function during development. However, very little is known about the target proteins of UBE3a, whose loss of regulation may directly contribute to the development of the Syndrome. In order to address this we have performed a number of proteomic analyses with a view to identifying critical interacting partners of UBE3a. We first demonstrate that UBE3a interacts directly with a number of proteasome subunits, and further, that Angelman Syndrome associated mutations within the UBE3a catalytic domain can have a profound inhibitory effect upon the normal functioning of the proteasome. This indicates that in certain cases, mutations within UBE3a has effects in addition to those associated with simple loss, implying a possible dominant negative function.
Further studies have also identified a diverse array of cellular proteins involved in the regulation of multiple cellular pathways, including vesicle transport and the Hippo signalling pathway. Current studies are dissecting which of these proteins are direct degradation or modification targets of the UBE3a, and provide potential novel targets for therapies aimed at rectifying the effects of UBE3a loss.

ABSTRACT N. 170

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**BARTESAGHI RENATA**	
Telethon grant N.	**GGP12149**	
Total budget €	**296.400**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2012**

PREVENTIVE THERAPY OF MENTAL RETARDATION IN DOWN SYNDROME BY A NOVEL GAMMA-SECRETASE INHIBITOR: FOCUS ON APP-DEPENDENT MECHANISMS IN NEURODEVELOPMENT

Calzà Laura (2), Magistretti Jacopo (3), Bartesaghi Renata (1)

(1) Dipartimento di Scienze Biomediche e Neuromotorie, University of Bologna, Piazza di Porta San Donato 2, 40126 Bologna, Italy, Tel +39.051.2091727, Fax +39.051.2091737, E-mail: renata.bartesaghi@unibo.it
(2) Dipartimento di Scienze Mediche Veterinarie, University of Bologna, Italy
(3) Dipartimento Di Biologia e Biotecnologie, University of Pavia, Italy

Down syndrome (DS) is a genetic pathology caused by triplication of human chromosome 21. Individuals with DS may have various medical problems, but intellectual disability is the unavoidable hallmark and the most invalidating aspect of this pathology. In spite of numerous efforts, the mechanisms whereby gene triplication leads to the DS phenotype have not been elucidated and there are no therapies to rescue brain developmental alterations and mental disability.
Generalised neurogenesis impairment during critical developmental stages and altered dendritic maturation appear to be major determinants of the reduced brain size and, hence, of mental retardation in individuals with DS. Recent data suggest that the trisomic gene APP (amyloid precursor protein), a gene important for neuron generation and differentiation, may be a key candidate for both these neurodevelopmental alterations in DS. This hypothesis derives from preliminary data in vitro showing that increased levels of AICD, a cleavage products of APP by g-secretase, impair proliferation of trisomic neural precursor cells and dendritic development. Since increased AICD levels in the DS brain are inevitable due to APP triplication, a rational therapeutic approach would be to prevent AICD formation by inhibiting the activity of APP g-secretase.
The overall goal of this project is to establish whether it is possible to pharmacologically cure impairment of brain development in DS by using a selective g-secretase inhibitor, in order to reduce AICD formation and restore neurogenesis and neuron maturation. We will use the Ts65Dn mouse model because it recapitulates numerous abnormalities of the DS brain. If, as we expect, this therapeutic approach will restore trisomy-linked brain alterations, results might open the way to clinical trials aimed at curing intellectual disability in children with DS.

ABSTRACT N. 171

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**PUSCH MICHAEL**	
Telethon grant N.	**GGP12008**	
Total budget €	**226.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

MOLECULAR MECHANISMS OF TRANSPORT, SMALL LIGAND MODULATION, AND SUBUNIT INTERACTION OF CHLORIDE TRANSPORTING CLC PROTEINS INVOLVED IN HUMAN GENETIC DISEASES

Fiore Michele, Gradogna Antonella, Jeworutzki Elena, De Stefano Silvia, Zanardi Ilaria, Zifarelli Giovanni, Pusch Michael

Istituto di Biofisica, CNR, Via De Marini 6, 16149 Genova, Tel. 010.6475561, Fax 010.6475500, E-mail: pusch@ge.ibf.cnr.it

CLC proteins are Cl ion transporting membrane proteins. Mutations in four of the nine CLC genes and in three associated small subunits cause various human genetic diseases.
Mutations in CLC-5 lead to Dent disease, characterized by low molecular weight proteinuria and kidney stones. We intend to investigate the mechanisms of transport coupling exploiting the properties of transport deficient mutants (e.g. E268A) which reveal properties of partial reaction paths. Furthermore, we intend to study the mechanism of rectification of CLC-5, a little understood property of this endosomal transporter. Here, we are aided by preliminary results on a point mutation which displays transport currents also at negative voltages. In particular, we intend to implement the cut-open oocyte voltage-clamp technique to improve the time resolution of the recordings.
Mutations in CLC-Kb and its beta-subunit barttin lead to Bartter syndrome, a salt wasting nephropathy associated with deafness in the case of barttin mutations. CLC-K channels are modulated by external Ca and protons. In this project we intend to characterize further the structure and function of a novel Ca binding site identified recently by us. We also will investigate an additional regulation of CLC-K channels at alkaline pH, discovered in preliminary experiments.
Recently, we have identified GlialCAM as a binding partner of CLC-2 in glial cells. Mutations in GlialCAM lead to megalencephalic leukoencephalopathy with subcortical cysts (MLC), a rare leukodystrophy characterized by macrocephaly. Interaction of GlialCAM with

CLC-2 leads to the clustering of CLC-2 at cell junctions and increases CLC-2 channel function. We intend to investigate the biophysical and cellular mechanism of the functional effects of GlialCAM on CLC-2.
The project targets the biophysical understanding of mechanisms that underlie various genetic diseases involving CLC channels/transporters. Such knowledge will aid in the development of treatments that are directed at increasing the function of these membrane proteins, either pharmacologically or genetically.

ABSTRACT N. 172

Telethon Research Projects - Neurological Diseases		
Principal Investigator	**RAGNINI-WILSON ANTONELLA**	
Telethon grant N.	**GGP08143**	
Total budget €	**222.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

MYOSIN-V-BASED HUMAN INHERITED DISEASES: MOLECULAR BASIS OF MYOSIN V RECOGNITION OF INTRACELLULAR CARGO

Frascella Annalisa (2,1), Mosca Maria Giovanna (1), Bau Cutrona Meritxell (1,2), Di Giandomenico Daniele (1), Bartocci Daniela (2,1), Porcu Giampiero (1), Ragnini-Wilson Antonella (1,2)

(1) Department of Cellular and Translational Pharmacology, Consorzio Mario Negri Sud Via Nazionale S.M. Imbaro (Ch), Italy, Tel. 087.2570317/412, E-mail: ragnini@negrisud.it
(2) Department of Biology, University of Rome Tor Vergata, Rome, Italy

MyoV-mediated organelle and vesicle motility is required for processes such as skin and hair pigmentation, immunological responses, myelination, and synaptic transmission and plasticity. Mutations at the C-terminus of brain MyoVa affect protein stability and are associated with Griscelli syndrome (GS) type 1. Pathological manifestations of GS1 include hypomelanosis with severe central nervous system dysfunction. The hypopigmentation defects are due to a failure in melanosome transport to melanocyte dendrites, a trafficking step that requires MyoVa and Rab27A interaction. How neurological manifestations of GS1 arise is unclear. In hippocampal neurons, MyoVa as well as MyoVb, by interacting with Rab11a, recycle endosomes that carry AMPA-type glutamate receptors at dendritic spines in response to Long Term Potentiation (LTP). This process is required for synaptic plasticity, learning and memory. In addition, recent data obtained in a MyoVa-null animal model show that MyoVa transports the ER into the dendritic spine of Purkinje cells [Hammer and Sellers 2012 Nature Mol.Cell Biol.13:1326]. Strikingly, while MyoVb is supposed to be the main motor mobilizing the AMPA receptor GluR1 subunit upon LTP stimulation at hippocampal neurons [Hammer and Sellers 2012 Nature Mol.Cell Biol.13:1326], its dysfunction causes microvillus inclusion disease (MVID), an autosomal recessive syndrome with no neurological implications.
To understand how many cargo transport defects are caused by MyoVa C-terminal mutations, such as those found in GS1 patients, it is necessary to determine the specificity of the interaction of each myosin V isoform. We showed previously that the interaction of the yeast MyoV, Myo2, with the Rab11 orthologue, Ypt32, requires a region of the MyoVs Globular Tail Domain (GTD), highly conserved from yeast to human [Casavola et al., 2008 Mol. Microbiol. 67:1051-66]. In the course of this project we used yeast and retinoic acid (RA)-differentiated SH-SY5Y neuroblastoma cells to show that MyoVa interacts with Rab11 in the vesicle binding region (VBR) of the MyoVa C-terminus. Moreover, using confocal and automated epifluorescence high-content microscopy analysis, we showed that mutation of the MyoVa VBR, overexpression of MyoVaGTD, and/or silencing of MyoVa causes AMPA receptor subunit GluR1 and Rab11 trafficking defects from soma to neurites in (RA)-differentiated SH-SY5Y cells. Furthermore, we established transfection and RA-differentiation protocols in 96-well plates suitable for high-throughput high-content image analysis of SH-SY5Y neuroblastoma cells to quantify GluR1 and Rab11 trafficking from the soma to neurites. This cell-based assay will be used to screen chemical/plasmid libraries for drugs/proteins that recover AMPA receptor subunit trafficking from the soma to dendrites in the presence of MyoVa loss-of-function using automated image analysis, as we described previously [Sacco et al., 2012 Mol Syst Biol. 8:603 doi:10.1038/msb.2012.36].

ABSTRACT N. 173

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**NICOLIS SILVIA KIRSTEN**	
Telethon grant N.	**GGP12152**	
Total budget €	**210.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF MOLECULAR TARGETS OF THE SOX2 TRANSCRIPTION FACTOR IN HUMAN INHERITED BRAIN DISEASE: AN APPROACH THROUGH SOX2 CONDITIONAL KNOCKOUT IN MOUSE

Favaro Rebecca (1), Mercurio Sara (1), Ferri Anna (1), Bertolini Jessica (1), Badiola Sanga Alexandra (1), Mariani Jessica (1), Beccari Leonardo (2), Zhang Yubo (3), Bovolenta Paola (2), Robson Paul (4), Wei Chia-lin (3), Nicolis Silvia (1)

(1) Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, piazza della Scienza 2, 20126 Milano, Italy, Tel. +39.02.64483339; Fax +39.02.64483314; E-mail: silvia.nicolis@unimib.it
(2) Centro de Biología Molecular Severo Ochoa. CSIC-UAM. Universidad Autónoma de Madrid, Madrid, Spain
(3) DOE Joint Genome Institute, Walnut Creek, CA, USA
(4) Genome Institute of Singapore, Singapore

Our project seeks to identify and functionally characterize molecular targets of the transcription factor Sox2, as mediators of its function in genetic brain disease. By Sox2 conditional ablation in mouse, we aim to identify genes regulated by Sox2, define Sox2 direct targets and their molecular mechanism of regulation by Sox2, and address their functional relevance for the pathological phenotype.
Sox2 heterozygous mutation in man causes a syndrome comprising a spectrum of nervous system defects. We previously reproduced essential aspects of this pathology by mouse genetic models of Sox2 deficiency. These showed that deficiency of one Sox2 target, the cytokine Shh, is critical for brain (hippocampal) pathology of these mice (also found in patients), as this was partially rescued by a drug mimicking Shh (Favaro R et al., Nat. Neurosci. 2009). More recently, we found that Sox2 ablation in early development (via a BF1-Cre transgene) causes major tissue loss in ventral telencephalon (striatum, GABAergic neurons), again depending on Shh; this reproduces aspects of holoprosencephaly, a disease that can be caused by SHH mutation. By a candidate-gene approach, we identified Nkx2.1, a known master regulator of ventral telencephalon development, and a direct regulator of Shh, as a novel Sox2 target gene. We also uncovered abnormalities in dorsal telencephalic (cerebral cortex/hippocampus) regions, that we are further characterizing.
We are also addressing Sox2 targets by genomic approaches. By RNAseq, we identified many Sox2-regulated genes by their differential expression in Sox2-ablated versus normal neural stem/progenitor cells (NSC). Many of these genes are bound, at their promoters, by Sox2. However, important DNA regulatory regions are often located at great distance from promoters of the regulated genes, and their location cannot be predicted based on proximity to the gene. We are addressing long-range interactions in the chromatin of normal, versus Sox2-ablated NSC by ChIA-PET (Handoko L et al., Nat. Genet. 2011). Sox2 ablation leads to a profound change in long-range interactions mediated by RNApolII, with both loss of many interactions, and generation of new ones. Many interactions involve genes differentially expressed by RNAseq, and many such genes are of known importance in the brain developmental processes impaired in our mutants. We are seeking to identify those long-range interactions that involve direct Sox2 binding (by available and new ChIPseq datasets), and will address their functional significance by transfection and transgenic studies.
The recent functional annotation of the human genome (nature.com/encode) revealed that a large proportion of SNPs associated to human genetic disease map within DNA regions of predicted regulatory significance (Maurano M et al, Science 2012). Identifying Sox2 targets will shed light on mechanisms of disease caused by Sox2, and may point to Sox2 involvement in diseases previously unrelated to Sox2.

ABSTRACT N. 174

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SANTORO MASSIMO**	
Telethon grant N.	**GGP10195**	
Total budget €	**338.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

MOLECULAR FUNCTIONS OF UBIAD1, A GENE PRODUCT ASSOCIATED TO SCHNYDER CRYSTALLINE CORNEAL DYSTROPHY

Vera Mugoni (1), Ruben Postel (2), Valeria Catanzaro (1), Elisa De Luca (1), Emilia Turco (1), Giuseppe Digiglio (1), Lorenzo Silengo (1), Micheal Murphy (3), Claudio Medana (4), Didier Stainier (5), Jeroen Bakkers (2), Massimo Santoro (1)

(1) Molecular Biotechnology Center, Università degli Studi di Torino, Via Nizza 52, 10126 Torino, Tel. 011.6706499/432
(2) Hubrecht Institute, University Medical Center and Interuniversity Cardiology Institute for the Netherlands, Utrecht, The Netherlands
(3) Medical Research Council Mitochondrial Biology Unit, Hills Road, Cambridge CB2 0XY, UK
(4) Department of Chemistry, University of Torino, Torino, Italy
(5) Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

Schnyder Crystalline Corneal Dystrophy (SCCD) is a rare genetic disease that causes progressive loss of visual acuity. Patients affected by SCCD show progressive accumulation of lipid molecules such as cholesterol and phospholipids in the cornea. The SCCD has been associated to dominant mutations in the human UBIAD1 gene but the molecular mechanisms of disease are still unknown.
By biochemical 13C-flux analysis we showed that UBIAD1 is a new non-mitochondrial prenyltransferase that catalyzes the biosynthesis of the antioxidant molecule CoenzymeQ10 (CoQ10). Subcellular fractionation experiments demonstrated that Ubiad1 performs its enzymatic activity in the Golgi membrane compartment. Lack of Ubiad1 induces cardiovascular oxidative damage both in a zebrafish model as well as in human primary endothelial cells. We demonstrated that Ubiad1 and Golgi-CoQ10 deficiency impairs NOS activity causing a NOS-dependent ROS production and consequent oxidative damage. SCCD-UBIAD1 mutations are able to protect from NOS-mediated oxidative stress both zebrafish embryos as well as human cells. Altogether our data uncover an intriguing genetic and metabolic role for UBIAD1 as antioxidant molecule in normal and SCCD pathogenesis. Maintenance of physiological CoQ10 and NO levels can be accounted for a role of Ubiad1 in SCCD. These findings might be used for SCCD diagnosis as well as development of therapeutic strategies.

ABSTRACT N. 175

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BONATTI STEFANO**	
Telethon grant N.	**GGP09029**	
Total budget €	**151.300**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2009**

ALPHA-B-CRYSTALLIN RESCUES CELL SURFACE EXPRESSION OF MUTANT FZ4 AND ATP7B RECEPTORS RETAINED IN THE ER

D'Agostino Massimo (1), Lemma Valentina (1), Caporaso Gabriella (1), Cannata Serio Magda (1), Chesi Giancarlo (2), Polishchuk Roman (2), Bonatti Stefano (1)

(1) Dipartimento di Biochimica e Biotecnologie Mediche, via S. Pansini 5, 80131 Napoli, +39.081.7463200, E-mail: bonatti@unina.it
(2) Telethon Institute of Genetics and Medicine (TIGEM), Napoli

Alpha-B-Crystallin (CRYAB) belongs to the small heat shock chaperone protein family and is characterized by an N-terminal domain that drives the formation of large oligomers, crucial for the chaperone function performed by the alpha-crystallin and C-terminal domains of the protein (Horwitz J., Exp. Eye Res. 76:145-153, 2003). Besides the lens, where it interacts with the alpha-Crystallin A chain to stabilize this remarkable structure, CRYAB has been found strongly expressed in brain, heart, muscle and kidney, and its chaperone activity has been demonstrated with a number of cytosolic proteins (Arrigo A.P. et al, FEBS Lett. 581:3665-3674, 2007) although it has been found as a cytosolic interactor of kidney cadherin-16, a plasma membrane single pass transmembrane protein (Thedieck C. et al., J. Mol. Biol., 145-153, 2008). We identified by proteomic analisis CRYAB as a partner of the human cell surface receptor Frizzled4 (Fz4) and its mutant form, retained in the endoplasmic reticulum (ER), which is associated to a dominant form of familial exudative vitreoretinophaty (adFEVR, Fz4-FEVR).
Interestingly, CRYAB over-expression, and not the mutant form R120G defective for the chaperone activity, rescued the cell surface localization of Fz4-FEVR and reverted its dominant negative effect on the cell surface expression of its wild-type counterpart (Kaykas A et al., Nat. Cell Biol., 6:52-58, 2006). Moreover, we found that CRYAB over-expression rescued the Golgi localization of the mutant form of ATP7B-H1069Q (Wilson Disease protein), which is similarly retained in the ER. Most attention has been dedicated to the chaperones of transmembrane proteins present in the lumen of the ER, but the cytosolic side of these complicate proteins may very well require assistance to reach proper folding.
Based on the property to resolve Desmin aggregates, our hypothesis is that CRYAB may inhibit the formation of large oligomeric complexes by Fz4-FEVR and ATP7B-H1069Q, thus favoring the attainment of correct folding. These results shed light on adFEVR and open the way to possible therapeutic strategies. They will contribute also to our understanding of the function of CRYAB, of protein transport along the secretory pathway and of Wilson Disease.

ABSTRACT N. 176

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SIMONELLI FRANCESCA**	
Telethon grant N.	**GGP10199**	
Total budget €	**221.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

A SAFETY AND EFFICACY STUDY IN SUBJECTS WITH LEBER CONGENITAL AMAUROSIS (LCA) USING ADENO-ASSOCIATED VIRAL VECTOR TO DELIVER THE GENE FOR HUMAN RPE65 TO THE RETINAL PIGMENT EPITHELIUM (RPE) [AAV2-HRPE65V2-301]: TREATMENT AND FOLLOW UP OF 3 ITALIAN PATIENTS

Testa Francesco (1), Rossi Settimio (1), Maguire Albert (2,3,4), Della Corte Michele (1), Di Iorio Valentina (1), Banfi Sandro (5,6), Surace Enrico Maria (5), Bennett Jean (2,3), Auricchio Alberto (5,7), Simonelli Francesca (1,5)

(1) Dipartimento di Oftalmologia, Seconda Università degli Studi di Napoli, Via S. Pansini 5, 80131, Napoli, Italy, Tel. +39.081.5666761, Fax +39.081.7704501; E-mail: francesca.simonelli@unina2.it
(2) Scheie Eye Institute, F.M. Kirby Center for Molecular Ophthalmology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA
(3) Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA
(4) University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA
(5) Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy
(6) Medical Genetics, Department of General Pathology, Seconda Università degli Studi di Napoli, Naples, Italy
(7) Medical Genetics, Department of Pediatrics, University of Naples "Federico II", Naples, Italy

Demonstration of safe and stable reversal of blindness after a single unilateral subretinal injection of a recombinant adeno-associated virus (AAV) carrying the RPE65 gene (AAV2-hRPE65v2) prompted us to determine whether it was possible to obtain additional benefit through a second administration of the AAV vector to the contralateral eye.
Re-administration of vector to the second eye was carried out in three adults with Leber congenital amaurosis due to mutations in the RPE65 gene 1.7 to 3.3 years after they had received their initial subretinal injection of AAV2-hRPE65v2.
Results (through 6 months) including evaluations of immune response, retinal and visual function testing, and functional magnetic resonance imaging indicate that readministration is both safe and efficacious after previous exposure to AAV2-hRPE65v2.

ABSTRACT N. 177

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**CARELLI VALERIO**	
Telethon grant N.	**GGP11182**	
Total budget €	**289.000**	
Centres: **4**	*Duration (yrs):* **2**	*Starting year:* **2011**

SYSTEMATIC GENE HUNTING FOR NUCLEAR MODIFIERS IN LEBER'S HEREDITARY OPTIC NEUROPATHY AND THEIR VALIDATION IN MODEL SYSTEMS

Carelli Valerio (1), Cantatore Palmiro (2), D'Adamo Pio (3), Daga Andrea (4)

(1) Dipartimento di Scienze Biomediche e NeuroMotorie, Università di Bologna, IRCCS Istituto delle Scienze Neurologiche, Via Altura 3, 40139 Bologna, Italy, Tel. +39.051.4966747, Fax +39.051.4966098, E-mail: valerio.carelli@unibo.it
(2) Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università di Bari
(3) Servizio di Genetica Medica, IRCCS Burlo-Garofolo, Università di Trieste
(4) IRCCS "E.Medea", Conegliano Veneto (TV)

Leber's hereditary optic neuropathy (LHON) is a blinding disorder affecting prevalently young males, recognized as the most frequent mitochondrial disease. LHON is associated with mtDNA point mutations in complex I subunit genes, but remains characterized by poorly understood features such as male prevalence, tissue specificity and incomplete penetrance. We undertook a multilayered "tour de force" to identify genetic modifiers in LHON by combining traditional genetic tools, such as linkage analysis, with high-throughput techniques, such as MitoExome sequencing, functional and tag SNPs genotyping and microarray expression studies. This effort is paralleled by an innovative strategy to generate complex I mutant mtDNA in Drosophila melanogaster, to provide a model of mtDNA-based complex I dysfunction.
We here schematically report the first year results as follows:
1. Linkage and SNPs genotyping studies in a large LHON pedigree. The linkage analysis failed to reach significant load scores under the assumption of a monogenic modifying trait. The subsequent genome wide association study generated a list of SNPs from which we selected the 50 top hits. These data indicate that penetrance in LHON is modulated by polymorphisms in different genes rather than by a single mutation. The use of co-variates (age, sex, smoke and mtDNA copy number assessed in blood cells) brought to a second list of candidate genes.
2. MitoExome in 27 discordant sib-pairs from 11 LHON families. We studied discordant male siblings (13 affected vs 14 unaffected carriers) for which the coding regions of 1605 mitochondrial proteins and the mtDNA itself were sequenced. No clear-cut association of any single variant emerged from this analysis. However, considering only the individuals belonging to the large family we selected 7 SNPs (2 protective and 5 risk). By including in the analysis the exposure to risk environmental factors (smoke and alcohol), 3 further variants were selected.
3. Microarry studies on muscle biopsies from discordant male siblings. We used 3 pairs of discordant male siblings from the previous group for which we had the skeletal muscle biopsy to run a microarray expression analysis. Four relevant candidate genes were obtained by this analysis.
4. Generation of complex I mutations in Drosophila mtDNA. We adopted a published strategy to select complex I mutations in mtDNA from Drosophila. We obtained four independent mutations affecting the ND5 subunit. These mutations are now screened for their pathogenic relevance. Testing the respiratory capacity of these mutants failed to reveal any impairment of mitochondrial respiration. More in depth analyses are being conducted to unveil any potential pathological feature.
In conclusion, the multi-layered approach of this project generated a number of candidate genes for a modifying role in LHON. The validation of the selected variants in large cohorts of LHON individuals is currently on the way.

ABSTRACT N. 178

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SCHIAFFINO MARIA VITTORIA**	
Telethon grant N.	**GGP08156**	
Total budget €	**384.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

DISSECTING THE MOLECULAR BASIS OF OCULAR ALBINISM TYPE 1: ENLIGHTENING SIGNAL TRANSDUCTION IN THE ENDOMEMBRANE SYSTEM

Palmisano Ilaria (1), Della Chiara Giulia (2), D'Ambrosio Rosa Lucia (1), Huichalaf Claudia (3), Brambilla Paola (4), Corbetta Silvia (2), Riba Michela (1), Piccirillo Rosanna (5), Valente Sergio (6), Casari Giorgio (1,7), Mai Antonello (6), Martinelli Boneschi Filippo (4), Gabellini Davide (3), Poli Guido (2,7), Schiaffino Maria Vittoria (1)

(1) Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy, Tel. +39-02-2643-4729; Fax +39-02-2643-6352; E-mail: schiaffino.mariavittoria@hsr.it
(2) Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan
(3) Dulbecco Telethon Institute and Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan
(4) Division of Neuroscience, San Raffaele Scientific Institute, Milan
(5) Department of Cell Biology, Harvard Medical School, Boston, MA, USA
(6) Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Roma
(7) Vita-Salute San Raffaele University, School of Medicine, Milan

Ocular albinism type 1 is an X-linked inherited disorder characterized by severe visual defects and by the presence melanosomal abnormalities in skin melanocytes and retinal pigment epithelium. The protein product of the ocular albinism gene, named OA1, is a G protein-coupled receptor exclusively localized to intracellular organelles (melanosomes and lysosomes) and necessary for the correct biogenesis and transport of melanosomes. In 2008, Lopez et al. (PLoS Biol, 2008, 6:e236) proposed that L-DOPA represents the endogenous ligand for OA1 and, among data in support, they reported that deprivation of a structurally related amino acid, namely tyrosine, determined the upregulation of an OA1 transgene expressed in COS7 cells by a plasmid vector. The authors attributed this result to the functional features of OA1 that in regular culture medium (which contains tyrosine) could bind the amino acid as a pseudo-ligand, being constitutively internalized and downregulated; by contrast, in tyrosine-free medium the receptor would recover its expression (Lopez et al., PLoS Biol, 2008, 6:e236). In order to establish the role of tyrosine in OA1 function, we performed a series of controls and surprisingly found that OA1 upregulation in tyrosine-free medium was neither specific for OA1 nor for tyrosine deprivation, but was also observed with other exogenous proteins and amino acids. Indeed, we established that, in the absence of essential amino acids, mammalian cells activate an epigenetic response, leading to the reactivation of silenced exogenous transgenes and retroviruses integrated into the genome (Palmisano I, et al. Proc Natl Acad Sci U S A, 2012, 109:E2284-93). This pathway was mediated in part by the specific downregulation of the histone deacetylase HDAC4 and was operational also in cells latently infected with HIV-1, the etiological agent of AIDS. Indeed, both amino acid starvation and pharmacological inhibition of HDAC4 promoted reactivation of HIV-1 transcription and virion production in HDAC4-positive ACH-2 T lymphocytic cells (Palmisano I, et al. Proc Natl Acad Sci U S A, 2012, 109:E2284-93). Thus, a serendipitous finding linked the biology of albinism to the mechanisms of epigenetic surveillance operated by mammalian cells to protect their DNA from the invasion of retroviruses and other parasitic nucleic acids. In addition to their biological relevance, our results suggest that selective targeting of HDAC4 might represent a novel strategy for modulating the expression of therapeutic viral vectors, as well as of integrated HIV-1 proviruses in latent reservoirs, without significant cytotoxicity.

ABSTRACT N. 179

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**LANZANI GUGLIELMO**	
Telethon grant N.	**GGP12033**	
Total budget €	**296.200**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2013**

DEVELOPMENT AND APPLICATION OF OPTO-NEURAL PROSTHETIC DEVICES AS A THERAPEUTIC APPROACH FOR RETINITIS PIGMENTOSA

Ghezzi Diego (2), Antognazza Maria Rosa (1), Maccarone Rita (3), Lanzarini Erica (1), Martino Nicola (1), Mete Maurizio (4), Pertile Grazia (4), Bisti Silvia (3), Benfenati Fabio (2), Lanzani Guglielmo (1)

(1) Department of Physics and Center for Nano Science and Technology, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133, Milano, Tel. 02.23999877; Fax. 02.23996126; E-mail: guglielmo.lanzani@iit.it
(2) Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Genova, Italy
(3) Dipartimento di Tecnologie Biomediche, Università dell'Aquila, L'Aquila, Italy
(4) UO Oculistica, Ospedale S. Cuore Don Calabria, Negrar (Verona), Italy

Interfacing soft organic materials and living neuronal tissues is at the forefront of bionanotechnology. To realize efficient retinal prosthesis, it is necessary to reliably transduce light into patterns of electrical activity in retinal networks. This multidisciplinary project aims at developing an efficient biocompatible photovoltaic interface for implantation in patients with Retinitis pigmentosa or related photoreceptor degenerative diseases.
The project aims at providing a proof-of-concept for the functionality of organic polymer-mediated photo-stimulation after in vivo implantation. Conventional silicon-based prosthesis (either passive or

with photodiodes) have a limited pixel resolution, are rigid and poorly tolerated. The device proposed here has many advantages with respect to the silicon-based ones which are currently on clinical testing, namely low heat production, much higher biocompatibility, no need of power supply, more intimate connection to living tissues thanks to the photo-transduction mechanism, enhanced spatial resolution, adjustable signaling timing. We recently discovered that primary neurons can be successfully grown onto a transparent photovoltaic organic polymer and electrically stimulated by light (Ghezzi et al., Nature Comm., 2011). Preliminary extracellular recordings of the retinal ganglion cell layer of degenerate retinas placed in subretinal contact with the polymeric film showed that a light stimulus much lower than the safe limit for pulsed illumination elicited intense activity. For these reasons, after a successful proof-of-concept in the animal, the device could be very close to a clinical application in selected patients of Retinitis pigmentosa.
We intend to: (i) improve the efficiency of the bio-organic interface by testing various biocompatible polymers, thicknesses of the active material, patterns of polymer deposition and by engineering of a biocompatible, flexible and porous substrate; (ii) investigate the light intensity- and wavelength-dependence of neural stimulation and the response to sustained patterned illumination by patch-clamp, MEA electrophysiology and functional imaging; (iii) analyze the light-response of normal and rod-degenerated (rd10 mice; Royal College of Surgeons (RCS) rats; light-degenerated albino rats) retinal explants; (iv) implant the device in subretinal configuration, in the degenerated rat/mouse eye and evaluate the tolerability and response to light.
We expect to set-up an efficient bio-organic photovoltaic interface to mimic the light sensitivity, transduction efficiency and spectral characteristics of an intact retinal networks. We also expect that this prosthesis activates retinal ganglion cell activity upon physiological illumination both in explants of degenerated retinas and in intraocular implants in experimental models of Retinitis pigmentosa. The realization of this bio-hybrid device will pave the way to a new generation of retinal prosthesis.

ABSTRACT N. 180

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FALSINI BENEDETTO**	
Telethon grant N.	**GGP10149**	
Total budget €	**141.400**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2011**

A NOVEL THERAPEUTIC STRATEGY TARGETING PHOTORECEPTOR OXIDATIVE DAMAGE IN ABCR-RELATED RETINAL DEGENERATIONS: AN EXPERIMENTAL AND CLINICAL STUDY

Falsini Benedetto (1), Piccardi Marco (1), Maccarone Rita (2), Bisti Silvia (2)

(1) Istituto di Oftalmologia, Università Cattolica del S. Cuore, Roma, Largo F. Vito 1, 00168 Roma, Tel 0630154929
(2) DISCAB (Dipartimento di Scienze Cliniche Applicate e Biotecnologiche), Università dell'Aquila, L'Aquila

Animal model (ABCA4 ko mice)
We studied the progression of degenerative process in control mice and in mice supplemented with saffron since they are born. We treated the mother (1mg/kg) and at wining we continued to treat each puppy separately. The aim was to verify whether and to what extent saffron treatment slows down the degenerative process. The main result was that while photoreceptor death progresses slowly the morphology of retina is highly affected and many markers of neuro-inflammation are expressed and modulated by saffron treatment. The dark adapted ERG response was maintained until two years of age but looked abnormal compared to controls. Mice are more resistant to light damage than normal control. Experiments are in progress for chronic exposure to light associated to saffron treatment "in vitro" to study in detail the properties and possible modifications of inner retinal circuitry. We are studying in treated and untreated animals the spatial organization of the receptive fields of ganglion cells whose axons form the optic nerve. Data analyses are in progress.
Clinical trial
The aim of this research is to investigate the influence of short-term Saffron supplementation on retinal function in STG/FF patients carrying ABCR mutations. In human patients, a randomized, double-blind, placebo controlled, cross-over study is currently ongoing. The macular cone-mediated electroretinogram (ERG) in response to high-frequency flicker (focal flicker ERG) is employed as the main outcome variable. To this end a submicrovolt technique has been specifically developed. The repeatability and reliability of this method was evaluated at baseline in 38 STD/FF patients with known ABCA4 genotype that have been enrolled in the study. fERGs were recorded from the macular region (18 deg) in response to a 41 Hz sinusoidally flickering uniform field on a light adapting background. Sixteen block averages (of 200 events each) for each replication were obtained for each eye. Two to four replications were obtained for each eye. fERG fundamental component was analyzed for each block to derive amplitude, phase and vector standard error (SE). Noise at the fundamental was estimated on signals averaged asynchronously at 1.1 times stimulus temporal frequency. Intra-test reliability was determined by taking into account either S/N ratio or vector SE. 96% of patients' responses differed significantly ($p < 0.05$) from noise. Lowest cut-off reliability, corresponding to a S/N ratio=3, was found in 7.7% of patients. The present fERG protocol in STDG/FF can provide a reliable macular function test with potential application for monitoring disease progression and treatment.
So far, the clinical trial (ClinicalTrials.gov Identifier: NCT01278277) has already enrolled 38 patients with known ABCA4 genotype and it is expected to end on May 2013.
References
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ABSTRACT N. 181

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GASPARINI PAOLO**	
Telethon grant N.	**GGP09037**	
Total budget €	**257.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

GENETICS OF HEARING LOSS

Girotto Giorgia, Vuckovic Dragana, Paolo Gasparini

Genetica Medica, IRCCS-Burlo Garofolo, Università di Trieste, Via dell'Istria 65, Trieste, Italy, Tel. +39.045.3785355, E-mail: geneticamedica@burlo.trieste.it

The main objective of the project is a comprehensive identification of the genetic causes of hearing loss (HL) to serve as targets for improved medical care of patients and their relatives. To reach this goal we have planned a strategy characterized by a combination of basic and applied research activities including the a) identification of new genes by high-throughput sequencing in a sample cohort, and by a genome-wide linkage/association approach in families and isolated populations, b) definition of molecular epidemiology data, new diagnostic guidelines and algorithms, and c) validation of a not invasive method for GJB2 carriers screening.
During the 3 years of the project the following activities have been carried out:
a) 3 GWAS on hearing quantitative traits by meta-analyzing data from 17 isolated populations of European and Central Asia ancestry for an overall number of 3800 individuals. Several significant loci (p<10-8 and p<10-7) were detected with a series of genes expressed within the inner ear. Data have also been used to construct highly significant "in silico" pathways for hearing function characterized.
b) 2 GWAS on hearing qualitative traits with a specific focus on age-related HL. Some significant (p<10-8 or p<10-7) were detected with a series of genes expressed within the inner ear. They are related with hearing development and hearing function, but also include genes whose function is still unknown. Some replicas have been carried out using other European Cohorts.
c) Matching the data obtained at point a and b of the present report we were able to define a list of strong candidate genes that have been chosen for validation investigating their expression by immunohystochemistry and by confocal microscopy using animal models. 3 genes show striking specific expression at the top of sensory hair cells in the cochlea, 1 is expressed in the marginal cells of the stria vascularis anwhile many others are clearly expressed throughout the cochlea.
d) Exome sequencing of 8 cases selected from patients identified within two independent isolated populations. Underlying causative gene and filtered data are now under analysis.
e) Exome sequencing of 6 Italian families (dominant inheritance) and 5 Qatari ones (recessive inheritance). For the Italian families 5 new loci/genes for which Sanger sequencing confirmation is now in

progress have been identified. 4 new loci and one new possible gene have been identified in Qatari families. In particular a causative mutation (c.7873 t>g leading to p.*2625Gluext*11) in BDP1 gene has been found in one family. The mutation disrupts the termination codon of the transcript resulting in an elongation of 11 residues of the BDP1 protein. Immunohistochemistry analysis carried out in the mouse inner ear showed Bdp1 expression in the mouse cochlea.
f) A validation phase of the rapid and not invasive method for GJB2 carriers screening by ultrasound means which confirmed the reliability of the protocol.

ABSTRACT N. 182

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MAMMANO FABIO**	
Telethon grant N.	**GGP09137**	
Total budget €	**331.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

EFFECTIVE GENE DELIVERY TO THE MOUSE INNER EAR VIA CANALOSTOMY

Crispino Giulia (1,2), Galindo Ramirez Fabian (2), Di Pasquale Giovanni (3), Jay Chiorini (3), Mammano Fabio (1,2,4)

(1) Fondazione per la Ricerca Biomedica Avanzata, Istituto Veneto di Medicina Molecolare, Via Orus, 2, 35129 Padova, Italy, Tel. 049.7923231, E-mail: fabio.mammano@unipd.it
(2) Dipartimento di Fisica "G. Galilei, Università" di Padova, Padova, Italy
(3) Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA
(4) Istituto CNR di Neuroscienze, Padova, Italy

Our recent studies have shown that in vitro gene delivery with recombinant bovine adeno associated virus (BAAV) vectors restores protein expression and rescues gap junction coupling in cochlear organotypic cultures from deaf, connexin-deficient mouse models (Ortolano et al., PNAS, 2008; Crispino et al., PlosOne, 2011).
In order to test BAAV efficacy also in vivo, we explored several routes for viral particles administration. Canalostomy in the semicircular canal is attractive for the relative simplicity of the surgical approach (Kawamoto, K., et al., Mol Ther., 2001) and also because it evolved from a labyrinthectomy used for the treatment of vertigo in humans. Using absorbance dyes and fluorescent markers, we confirmed that canalostomy minimizes injury to existing structures and ensures distribution of the injected agent throughout the cochlea.
We then used this technique to deliver BAAV vectors containing a Cre–IRESGFP expression cassette (BAAVCre–IRESGFP) to the inner ear of P4 and P30 Cx26loxP/loxP mice. Cre recombinase expression in vivo catalyzed recombination of the floxed Cx26 gene ensuing in a ~50% reduction of Cx26 mRNA level. Three weeks after injection, hearing thresholds, obtained by recording auditory brainstem responses (ABRs), were significantly elevated in Cx26loxP/loxP mice that had received BAAVCre–IRESGFP but remained normal in siblings injected with vehicle alone, as well as in wild type mice injected with Cre-IRESGFP BAAV.
Next we tried to restore hearing function in deaf Cx30 knock out mice. Previous studies demonstrated that overexpression of Cx26 by BAC insertion completely rescued hearing in Cx30 KO mice (Ahmad S. et al., PNAS, 2007). In our study, we delivered Cx26CFP BAAV by canalostomy in P4 Cx30 KO deaf mice. Three weeks after surgery we recorded ABR and observed unchanged hearing thresholds. In order to understand this result, we analyzed Cx26CFP mRNA level at different time points after BAAV injection. We observed a maximal Cx26CFP expression 2 days after surgery, that decreased significantly day by day, disappearing almost completely 30 days after surgery.
Altogether, our results clearly demonstrated that: 1) BAAV transduces the mouse inner ear in vivo efficiently but with transient effect and 2) connexin expression is necessary for auditory function at any age.
Our next purpose will be to engineer BAAV vectors to permit a stable expression of recombinant connexin in the inner ear and permit a rescue of hearing.

ABSTRACT N. 183

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**DUGA STEFANO**	
Telethon grant N.	**GGP11177**	
Total budget €	**345.000**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

OMICS-BASED APPROACHES FOR THE IDENTIFICATION OF NOVEL INHERITED NON-SYNDROMIC SENSORINEURAL HEARING LOSS-RELATED GENES

Soldà Giulia (1), Robusto Michela (1), Asselta Rosanna (1), Ghilardi Anna (2), Castorina Pierangela (3), Benzoni Elena (3), Primignani Paola (4), Ambrosetti Umberto (3), Del Giacco Luca (2), Duga Stefano (1)

(1) Dipartimento di Biotecnologie Mediche e Medicina Traslazionale (BIOMETRA), Via Viotti 3/5, 20133, Milano, Italy, Tel. 02.50315823, Fax 02.50315864, E-mail: stefano.duga@unimi.it
(2) Dipartimento di Bioscienze, Università degli Studi di Milano, Milano, Italy
(3) Laboratorio di Genetica Medica e UO Audiologia, Fondazione IRCCS, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milano, Italy
(4) S.O. Genetica Medica, Ospedale Niguarda Cà Granda, Milano, Italy

Objectives
This project is aimed at identifying novel genetic determinants of non-syndromic sensorineural hearing loss (NSHL) by a double approach, consisting in: a) investigating the pathogenic mechanisms of mutations within the MIR96 gene, and b) searching for new genes/mutations by Whole-Exome Sequencing (WES) in selected NSHL families and their validation in zebrafish.
Obtained results
a) MiR-96 is part of the conserved miR-183 microRNA (miRNA) family, which plays essential functions in the vertebrate inner ear. Point mutations within the seed region of miR-96-5p cause autosomal dominant NSHL (AD-NSHL). We identified a novel mutation, miR-96(+57T>C), within MIR96 in an Italian AD-NSHL family. The mutation replaces a highly conserved nucleotide and is predicted to reduce the stability of the pre-miRNA hairpin. Ex-vivo assays in mammalian cells confirmed that both miR-96-5p and miR-96-3p mature species were significantly reduced in the mutant, whereas the precursor level was unaffected. Moreover, miR-96-5p and miR-96-3p expression could be restored to normal levels by reconstituting the secondary structure of the pre-miR-96 hairpin, thus demonstrating that the mutation hinders the precursor processing. Finally, even though the mature miR-96-5p sequence is not altered, we demonstrated that the identified mutation significantly impacts on miR-96-5p regulation of selected targets.
Taken together, these data provide further evidence of the involvement of miR-96 in human deafness and demonstrate that a quantitative defect of this miRNA may contribute to NSHL.
b) Four genetically undiagnosed Italian families (NSHL1-4) with recessive NSHL were analyzed by WES. For 3 families (NSHL1-3), no putative pathogenic mutations in known NSHL genes were found. Candidate variants in novel genes are currently being tested to evaluate their segregation with the disease in the probands' families and their recurrence in a cohort of 1,124 Italian NSHL patients. In parallel, the Zebrafish unit is setting up pilot knock-down/overexpression experiments (by testing a known deafness gene, SMPX), which will be instrumental to functionally validate novel genes/mutations.
In family NSHL4, sequencing of a single proband lead to the identification of a novel missense mutation within the PRPS1 gene (DFNX1 locus), which codes for the phosphoribosylpyrophosphate synthetase 1 (PRS-I) enzyme. A subsequent screening of PRPS1 by Sanger sequencing in 13 additional unrelated X-linked NSHL families led to the discovery of a second missense mutation segregating with pre-lingual hearing impairment. Both amino acid substitutions are predicted to destabilize the enzyme structure and result in a marked reduction (>60%) of PRS-I activity in the patients' erythrocytes compared to controls. In conclusion, our data highlight the recurrence of PRPS1 mutations among Italian NSHL patients, suggesting that it represents a major locus for X-linked NSHL to be included in genetic screenings.

ABSTRACT N. 184

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**IOLASCON ACHILLE**	
Telethon grant N.	**GGP09044**	
Total budget €	**310.100**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

REGULATORY NETWORK GATA1-MEDIATED OF SEC23B GENE: IMPLICATIONS FOR THE DIAGNOSIS OF CONGENITAL DYSERYTHROPOIETIC ANEMIA II

Russo Roberta (1,2), Andolfo Immacolata (1), Gambale Antonella (1), Esposito Maria Rosaria (1), Langella Concetta (1,2), Asci Roberta (1), Iolascon Achille (1,2)

(1) CEINGE, Biotecnologie Avanzate, Via Gaetano Salvatore 486, 80145 Napoli, Tel. +39.081.3737898, Fax +39.081.3737804, E-mail: achille.iolascon@unina.it
(2) Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Napoli

SEC23B gene encodes for a component of the coat protein complex (COP)II, involved in the anterograde protein trafficking endoplasmic reticulum-Golgi. Mutations in this gene cause the Congenital Dyserythropoietic Anemia II (CDAII), an autosomal recessive disorder with ineffective erythropoiesis. Almost 60 different causative mutations have been described (Iolascon, 2011; Russo, 2011; Punzo, 2011; Liu, 2012). The vast majority of patients has two mutations according to the pattern of recessive inheritance. However, in our cohort about 10% of cases showed only one SEC23B mutation in heterozygous state. So, we postulated the occurrence of a second mutation in GATA1 gene, assuming that it could be involved in the regulation of SEC23B expression. GATA1 transcription factor is a key regulator of the erythro- and thrombopoiesis. Indeed, some mutations in this gene have been already associated to a specific CDA variant, X-linked dyserythropoietic anemia and thrombocytopenia (XDAT).
Our aims were (i) to perform a mutational screening of GATA1 genomic sequence in CDAII cases negative for SEC23B or with incomplete pattern of mutations; (ii) to characterize the promoter region of SEC23B and (iii) to study GATA1-mediated regulation of SEC23B expression.
GATA1 mutational screening was performed according to previous method (Russo, 2010). We found only one XDAT patient with the hemizygous mutation Gly208Arg. Two CDAII patients, with incomplete SEC23B pattern, showed the same polymorphic variant (rs113966884 G/A) in GATA1 5'upstream region. We demonstrated that both variations result in a reduced GATA1 expression which in turn correlate with a decrease of SEC23B, implying a functional correlation between the two genes.
Promoter analysis is an essential step on the way to identify regulatory networks. In order to characterize SEC23B promoter region, we established 10 deletion mutants covering a 3500 bp upstream region of the gene. By luciferase assay of the deletion mutants we identified the minimal promoter region of the SEC23B gene. In silico analysis of SEC23B upstream region predicted the presence of putative binding sites of the main transcription factors (TFBS) involved in the erythroid differentiation, GATA1 and KLF1: indeed, mutations in both proteins cause peculiar type of inherited dyserythropoietic anemias. The GATA1-mediated regulation of SEC23B expression has been confirmed by co-transfection of pCDNA3.1-GATA1/G208R and pGL3-HuSEC23B/3.44 constructs in HEK-293 cell line. The effective binding of GATA1 to SEC23B promoter region has been proven by chromatin immuno-precipitation (ChIP) assay in erythroid cellular model.
The identification of TFBS in SEC23B promoter could allow the definitive diagnosis of CDAII patients with peculiar clinical phenotypes or those with incomplete pattern of mutations in SEC23B. This study also provides new insights in the molecular mechanisms of SEC23B regulation.

ABSTRACT N. 185

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**PINOTTI MIRKO**	
Telethon grant N.	**GGP09183**	
Total budget €	**345.300**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2009**

RNA-BASED THERAPEUTIC APPROACHES FOR BLOOD COAGULATION FACTOR DEFICIENCIES CAUSED BY SPLICING MUTATIONS

Balestra Dario (1), Fernandez Eugenio (2), Dal Mas Andrea (2), Cavallari Nicola (1), Barbon Elena (1), Bussani Erica (2), Ferraresi Paolo (1), Canella Alessandro (1), Branchini Alessio (1), Campioni Matteo (1), Baroni Marcello (1), Mattioli Chiara (2), Pagani Franco (2), Pinotti Mirko (1)

(1) Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, Via Fossato di Mortara 74, 44121 Ferrara, Tel. 0532.424424, Fax 0532.424484, E-mail: pnm@unife.it
(2) Human Molecular Genetics, International Centre for Genetic Engineering and Biotechnology, Padriciano, Trieste

Aberrant pre-mRNA splicing induced by mutations is a frequent cause (>20%) of severe coagulation factor VII (FVII, autosomal) and IX (FIX, hemophilia B, X-linked) deficiencies.
FVII/FIX deficiencies may lead to life-threatening bleeding and their treatment still presents several drawbacks. Since restoring even low FVII/FIX levels (>3%) can guarantee haemostasis, these diseases represent ideal models to test innovative therapeutic approaches.
PATHOLOGICAL SPLICING MECHANISMS AND RESCUE OF FVII/FIX LEVELS BY MODIFIED U1snRNA IN CELLULAR MODELS - Through specific F7/F9 minigene splicing assays we established the disease-causing mechanism of 25 different mutations at donor splice sites (5'ss), which resulted either in exon skipping or cryptic 5'ss activation. We demonstrated that the key spliceosomal component U1snRNA (U1 small nuclear RNA), engineered to bind by complementarity to mutated 5'ss, efficiently corrected several 5'ss defects in F7/F9 genes. Intriguingly, these U1snRNAs also corrected some acceptor splice site (3'ss) mutations. Through splicing-competent cDNA constructs we showed that U1snRNA-mediated splicing correction resulted in remarkable rescue of secreted FVII/FIX protein levels with coagulant activity (from undetectable to >10% for FVII, up to 100% for FIX).
To improve specificity for F9 gene, we designed U1snRNAs targeting non-conserved intronic sequences downstream of the 5'ss (Exon Specific U1snRNA, ExSpeU1). We found a gradient of rescue efficacy, which decreased with the 5'ss distance. Intriguingly, the best ExSpeU1 (ExSpeU1-FIX+9) remarkably rescued FIX biosynthesis and function (~90%) impaired by different 5'ss or 3'ss mutations.
RESCUE OF FVII LEVELS IN ANIMAL MODELS - In cellular models, the modified U1snRNA U1+5a restored human FVII (hFVII) function impaired by the 5'ss mutation c.840+5G>A. We assessed the U1+5a-mediated rescue in mice, either by transient (by non-viral vectors) or prolonged (by adeno-associated viral [AAV] vectors) co-expression of the mutated splicing-competent hFVII minigene (FVII+5A) and of the U1+5a, driven by separate vectors. In both experimental systems, correct hFVII transcripts were detectable in hepatocytes. Transient U1+5a co-expression resulted in hepatocyte localization of hFVII and secreted levels up to 367 ng/ml (~17% of wt-hFVII). Prolonged and vector dose-dependent hFVII expression in plasma (from undetectable up to 30 ng/ml) was demonstrated via delivery of AAV-FVII+5A and AAV-U1+5a viral vectors.
CONCLUSIONS - For the first time, we demonstrated that a unique ExSpeU1 can restore gene expression impaired by various splicing mutants, and the increase of coagulant FVII/FIX levels, if achieved in patients, would be far beyond the therapeutic threshold. Moreover, we provided the first in vivo proof-of-principle of the therapeutic potential of the U1snRNA-mediated correction of splicing mutations, a frequent cause of all human genetic diseases.

ABSTRACT N. 186

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FOLLENZI ANTONIA**	
Telethon grant N.	**GGP09280**	
Total budget €	**125.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

THERAPEUTIC ROLES OF HEALTHY DONOR HUMAN LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSEC), BONE MARROW OR CORD BLOOD-DERIVED CELLS IN HEMOPHILIA A

Merlin Simone (1), Zanolini Diego (1), Ranaldo Gabriella (1), Cannizzo Stefania (1), Feola Maria (1), Valente Guido (2), Prat Maria (1), Follenzi Antonia (1)

(1) Università del Piemonte Orientale, Dipartimento di Scienze della Salute, via Solaroli 17, 28100, Novara, Tel. 0321.660674, E-mail: antonia.follenzi@med.unipmn.it
(2) Università del Piemonte Orientale, Dipartimento di Medicina Traslazionale, Novara

Identification of cells capable of synthesizing and releasing factor VIII (FVIII) is critical for insights into pathophysiological mechanisms and for developing therapeutic approaches in hemophilia A. Endothelial cells, particularly liver sinusoidal endothelial cells (LSEC), express FVIII most in the body. However, recent studies of bone marrow (BM) transplantation suggested additional cell types could synthesize and release FVIII, and also correct bleeding in hemophilia A mice (Follenzi et al., 2012). Therefore, to establish the ability of circulating blood cells in expressing FVIII, we analyzed several murine and human hematopoietic cell types. First, we generated polyclonal antibody against recombinant human (h) FVIII. The mono-specificity of this FVIII antibody was established by western blotting. Second, we found by immunostaining that FVIII was present in human hematopoietic cells isolated from peripheral blood or BM, as well as in cells from h cord blood (CB). These peripheral blood, BM and CB cell types, included myeloid cells, e.g., monocytes, macrophages, dendritic cells, and megakaryocytes. The identity of these cell types was verified by costaining for FVIII and cell type-specific markers, e.g., CD14 for monocytes/macrophages, CD11c for dendritic cells, and vWF or CD61 for megakaryocytes differentiated from c-Kit+ BM cells. Moreover, FVIII expression in these cell types was verified by RT-PCR at the mRNA level and by western blot at the protein level. Third, antibody staining confirmed FVIII expression in normal h liver, including in LSEC, Kupffer cells (KC), and hepatocytes. Also, we observed FVIII expression in mononuclear or endothelial cells in other organs, e.g., spleen lungs and kidneys. Finally, transplantation studies were performed in hemophilia A mice with either wild-type mouse KC or immunoselected CD11b+ monocytes in human CB. Tissue analysis with immunostainings showed transplanted cells entered the mouse liver and survived for at least 1 week. Tail clip challenge in hemophilia A mice showed correction of bleeding in 9 of 14 mice (64%) after KC, and 9 of 11 mice (82%) after cells from human CB. Transplantation studies with h cells were done in hemophilia mice in NOD/SCID immunodeficient background. We confirmed by aPTT and COATEST assays presence of plasma FVIII activity in several surviving hemophilia A mice. Recently, we started to process human liver biopsy to isolated hepatocytes, LSEC and KC and we confirmed FVIII expression in these cell types. To verify the potential proliferating capacity of LSEC, we started in vitro cultures of them and we plan to transplantion in hemophila A gamma-null mice to verify the therapeutic efficacy.
Conclusions: Besides human endothelial cells from livers, FVIII was expressed in circulating blood cells, including monocytes, macrophages and megakaryocytes. These insights in additional cellular origins of FVIII offer new mechanisms for understanding alterations in FVIII synthesis and release for developing therapies in hemophilia.

ABSTRACT N. 187

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TOLOSANO EMANUELA**	
Telethon grant N.	**GGP12082**	
Total budget €	**180.800**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

THERAPEUTIC STRATEGIES TO AMELIORATE HEME-DRIVEN TISSUE OXIDATIVE INJURY IN SICKLE CELL ANEMIA

Vinchi Francesca, Ingoglia Giada, Petrillo Sara, Chiabrando Deborah, Fiorito Veronica, Mercurio Sonia, Tolosano Emanuela

Molecular Biotechnology Center, Via Nizza 52 10126 Torino, Tel. 011.6706423, Fax 011.6706432,; E-mail: emanuela.tolosano@unito.it

Sickle cell disease (SCD) is characterized by enhanced intravascular hemolysis which results in heme-catalyzed reactive oxygen species generation, leading to endothelial activation and tissue damage. The depletion of the plasma hemoglobin and heme scavengers, Haptoglobin and Hemopexin, and the overexpression of the heme degrading enzyme Heme Oxygenase-1 indicate that the physiologic anti-oxidant response in SCD patients is exhausted and suggest that drugs able to limit heme toxic effects may be beneficial. Accordingly, we have already demonstrated that administration of Hemopexin ameliorates the health status of sickle mice by reducing oxidative tissue injury, endothelial activation and inflammation. This was achieved by promoting, other than hepatic heme catabolism, also heme excretion in the bile.
This project is aimed at ascertaining whether the enhancement of heme excretion in the bile may represent a therapeutic strategy to prevent heme-driven tissue injury in SCD. We will focus on the role of the heme exporter Feline Leukemia Virus subgroup C Receptor (FLVCR1a) that is the strongest candidate to mediate heme export from hepatocytes to bile canaliculi. We will take advantage from genetic models already available in our laboratory, i.e. sickle mice and liver specific FLVCR1a-null mice to study the role of FLVCR1 in heme detoxification in SCD.
The specific aims of this project are:
1) To study FLVCR1 localization in hepatocytes;
2) To evaluate the role of FLVCR1 during acute and chronic hemolysis and in SCD;
3) To establish and validate a system for enhancing FLVCR1 expression in the liver;
4) To evaluate the effects of a combined therapy that promotes hepatic heme detoxification, for sickle cell anemia.

Results
The analysis of liver specific FLVCR1a-null mice under basal conditions demonstrated that FLVCR1a-mediated heme effux is crucial to prevent heme-iron loading and oxidative stress. The differences between liver specific FLVCR1a-null mice and wild-type animals were exacerbated after acute heme overload that, consistently, caused a significant increase in FLVCR1a expression in the liver of wild-type mice. On the other hand, FLVCR1a was down-regulated in the liver of wild-type mice after chronic heme overload suggesting that FLVCR1a-mediated heme export out of hepatocytes has to be blocked when plasma heme level is constantly high. Future studies are needed to elucidate this point.
In this project, we expect to find a way to efficiently promote heme detoxification thus preventing heme-driven activation and vascular damage. This might improve the current therapies based on iron chelation and not-specific anti-oxidant drugs.**-**
[Vinchi et al., Hemopexin therapy inhibits heme-driven endothelial activation and inflammation in mouse models of hemolytic diseases via the induction of hepatic Heme Oxygenase activity, Circulation, under revision]

ABSTRACT N. 188

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GAMBARI ROBERTO**	
Telethon grant N.	**GGP10214**	
Total budget €	**132.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

HEMOGLOBIN PRODUCTION IN BETA-THALASSEMIA ERYTHROID CELLS FOLLOWING ALTERATION OF BIOMOLECULAR PATHWAYS REGULATING GLOBIN GENE EXPRESSION

Bianchi Nicoletta (1,2), Borgatti Monica (1,2), Zuccato Cristina (1,2), Finotti Alessia (1,2), Breveglieri Giulia (1,2), Salvatori Francesca (1,2), Breda Laura (3), Gardenghi Sara (3), Brognara Eleonora (1,2), Lampronti Ilaria (1,2), Fabbri Enrica (1,2), Fibach Eitan (4), Rivella Stefano (3), Gambari Roberto (1,2)

(1) Dipartimento di Biochimica e Biologia Molecolare, Via Fossato di Mortara 74, Ferrara, Italy, Tel. 0532.974443, Fax 0532.974500; E-mail: gam@unife.it
(2) Dipartimento di Scienze della Vita e Biotecnologie, Ferrara, Italy
(3) Weill Cornell Medical College, Department of Pediatrics, Division of Hematology-Oncology, New York, USA
(4) Hadassah Hospital, Jerusalem, Israel

Overall objectives.
Aim 1 - recruitment and genetic characterization of beta-thalassemia patients.
Aim 2 - characterization of molecular mechanisms of action of fetal hemoglobin (HbF) inducers and their use in combination with gene therapy employing lentiviral vectors.
Aim 3 - study of gene expression in HPFH (hereditary persistence of fetal hemoglobin) patients and in beta-thalassemia patients producing very high levels of HbF.
Aim 4 - analysis of the microRNA network in HPFH cells and in erythroid precursor cells (ErPCs) induced to HbF production. Aim 5 - application of the read-through approach on adult hemoglobin (HbA) production by beta°39 thalassemic cells.
Results.
I. We have participated in the development of a novel therapeutic vector (Ank-T9W) for therapy of beta-thalassemia and confirmed that a combined treatment of ErPCs from homozygous beta°39 and IVS-110 beta-thalassemic patients with the HbF inducer mithramycin (MTH) and lentiviral vectors leads to HbF/HbA induction fully reducing the excess of alpha-globin, suggesting a combination of gene therapy and HbF induction (GT/HbF strategy) for treatment of beta-thalassemia.

II. We have further studied the link between expression of microRNA 210 and induction of erythroid program and HbF induction in erythroid cells treated with MTH. We have confirmed in raptor mRNA a possible target site of microRNA 210. MTH-mediated induction of HbF is associated with inhibition of raptor gene expression and mTOR-C1 pathway.
III. We have developed novel K562 cell clones harbouring BCL11A sequences for studying activation of HbF production following interference with repressors of gamma-globin gene transcription. We have studied BCL11A during HbF induction of erythroid precursor cells (ErPC) from beta-thalassemia patients treated with MTH and found that increase of gamma-globin gene expression and HbF production is preceded by a sharp decrease of BCL11A levels in ErPC from beta-thalassemia patients treated with MTH. These results suggest that BCL-11A levels are associated with gamma-globin gene expression. Therefore, disrupting the bindings of the BCL-11A transcriptional complex to the gamma-globin gene promoter provides a novel approach for inducing expression of the gamma-globin genes.
IV. We have confirmed read-through in ErPCs from homozygous beta°39-thalassemia patients, in spite of the low level of adult hemoglobin associated with the decreased abundance of the beta°39-globin mRNA subjected to non-sense mediated decay (NMD). We have developed K562 cell clones with silenced UPF-1 sequences.
V. We have developed a novel PCR-independent protocol for molecular diagnosis of beta-thalassemia based on SPR-Imaging, PNA probes and nanoparticle enhancers.
VI. We have further characterized mouse transgenic lines carrying the beta-thalassemia beta°39, IVS-I-6, e IVS-I-110.

ABSTRACT N. 189

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GRESELE PAOLO**	
Telethon grant N.	**GGP10155**	
Total budget €	**124.300**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

CONSTITUTIVE ACTIVATON OF ALPHAIIB-BETA3 RECEPTOR DUE TO AN INHERITED MUTATION OF INTEGRIN BETA3 LEADS TO DEFECTIVE PLATELET FUNCTION AND IMPAIRED THROMBOPOIESIS

Bury Loredana (1), Cecchetti Luca (1), Giannini Silvia (1), Corazzi Teresa (1), Appolloni Viviana (1), Guglielmini Giuseppe (1), Falcinelli Emanuela (1), Mezzasoma Anna Maria (1), Savoia Anna (2), Malara Alessandro (3), Balduini Alessandra (3), Italiano Joseph E (4), Gresele Paolo (1)

(1) Dipartimento di Medicina Interna, Sezione di Medicina Interna e Cardiovascolare, Università di Perugia, Via Enrico dal Pozzo, 06126 Perugia, Italia, Tel. 075.5783989, E-mail: grespa@unipg.it
(2) Laboratory of Genetics, IRCCS Burlo Garofolo, Trieste, Italy
(3) Biotechnology Laboratories, Department of Biochemistry, University of Pavia, Italy
(4) Dep. Of Medicine, Hematology Division, BWH Hospital, Boston MA, USA

Mutations of integrin alphaIIb-beta3 lead to Glanzmann Thrombasthenia (GT), an autosomal recessive bleeding disorder characterized by absent platelet aggregation but a normal platelet count and volume. We have recently described a novel autosomal dominant hereditary macrothrombocytopenia with platelet dysfunction and a mucocutaneous bleeding diathesis associated with a heterozygous mutation (2134+1 G>C) of the ITGB3 gene, coding for the beta3 subunit of the alphaIIb-beta3 receptor (Gresele P. et al. Haematologica. 2009;94:663-9): a variant form of GT.
Studies in a model of the mutation expressed in CHO cells we developed reveal that the mutation exerts a dominant negative effect and induces constitutive activation of the alphaIIb-beta3 receptor. The co-expression of the mutant beta3 subunits together with the normal protein decreases the number of alphaIIb-beta3 receptors expressed on the cell surface, as shown by flow cytometry and Western blotting. Mutant integrin-bearing CHO cells bind fibrinogen and PAC-1 without the need of stimulation and aggregate spontaneously in the presence of fibrinogen. Western blotting shows constitutively phosphorylated beta3 cytoplasmic tail and Focal adhesion kinase in CHO cells expressing the mutant beta3 subunit, spreading on fibrinogen is faster while clot retraction is defective, results compatible with defective alphaIIb-beta3 mediated outside-in signalling. Patients' platelets show constitutive activation of outside-in signalling leading to increased actin polymerization in resting platelets and spontaneous spreading on Von Willebrand factor.
To unravel the cause of macrothrombocytopenia we studied megakaryocytopoiesis and pro-platelet formation by isolating hematopoietic stem cells from patient's peripheral blood and culturing them until differentiation in megakaryocytes. Moreover, we expressed the mutant alphaIIb-beta3 receptor in fetal liver-derived murine megakaryocytes. Spreading on different extracellular matrix proteins and proplatelet formation were analyzed. Megakaryocyte maturation was normal but proplatelets were abnormal, with tips decreased in number and larger in size than those of controls. Abnormal megakaryocyte spreading on fibrinogen was observed, with 50% of spread cells showing disordered actin distribution and focal adhesion points more evident than stress fibers. Integrin alphaIIb-beta3 expression on megakaryocytes was reduced but the receptor was constitutively activated, as shown by a sustained and substrate-independent activation of Focal Adhesion Kinase. In addition, platelet maturation from preplatelets was impaired, generating platelets of heterogeneous dimensions.
Taken together these data show that an outside-in signaling generated by a constitutively activated alphaIIb-beta3 receptor is the cause of both macrothrombocytopenia and defective platelet function in our GT variant.

ABSTRACT N. 190

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BALDUINI CARLO LUIGI**	
Telethon grant N.	**GGP10089**	
Total budget €	**373.000**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2011**

A NEW GENE FOR INHERITED THROMBOCYTOPENIAS: CLINICAL, PATHOGENETIC AND PHARMACOLOGICAL STUDIES

Perrotta Silverio (2), Seri Marco (3), Savoia Anna (4), Balduini Carlo (1)

(1) Medicina Interna III, Fondazione IRCCS Policlinico San Matteo-Università degli Studi di Pavia, Piazzale Golgi, 27100 Pavia, Tel. 0382.502183, Fax 0382.526223, E-mail: c.balduini@smatteo.pv.it
(2) Dipartimento di Padiatria, Seconda Università di Napoli, Napoli
(3) U.O. Genetica Medica, Dipartimento di Scienze Mediche e Chirurgiche, Università degli Studi di Bologna, Policlinico S. Orsola-Malpighi, Bologna
(4) Dipartimento di Scienze mediche, chirurgiche e della salute, Università di Trieste, IRCCS Burlo Garofolo, Trieste

OVERALL OBJECTIVES OF THE STUDY
Unravel etiopathogenesis of thrombocytopenia 2 (THC2, OMIM 188000), describe its clinical features and identify molecules for its treatment.
SPECIFIC AIMS
1) Etiopathogenesis: characterization of molecular and cellular abnormalities.
2) Clinical features: description of clinical and laboratory pictures of affected subjects.
3) Therapy: identification of drugs that could be used to improve thrombocytopenia.
RESULTS
Molecular abnormalities. The study of 35 affected subjects belonging to 9 families with THC2 allowed us to identify 6 different mutations in a highly conserved 19 bp sequence located in the 5' untranslated region (UTR) of ANKRD26, a gene whose functional role is unknown. These variants, which always segregated with the thrombocytopenia along the pedigrees, were not found in 500 controls, nor were they reported in the 1000 Genomes database. The luciferase reporter assay suggested that these 5' UTR mutations enhance ANKRD26 expression (Pippucci et al. Am J Hum Genet 2011;88:115-20).
Clinical features. The analysis of our case series of 210 families with inherited thrombocytopenias allowed us to identify 21 families with mutations in the 5'UTR of ANKRD26. THC2 is therefore one of the less rare forms of inherited thrombocytopenias. Thrombocytopenia and bleeding tendency were usually mild. Nearly all patients had no platelet macrocytosis, and this characteristic distinguishes THC2 from most of the other forms. In the majority of cases in vitro platelet aggregation was normal. Bone marrow examination and serum thrombopoietin levels suggested that thrombocytopenia was derived from dysmegakaryopoiesis. Unexplained high values of hemoglobin and leukocytes were observed in some cases. An important finding was an incidence of acute leukemia much higher than expected (Necchi V et al. Thromb Haemost, in press).
Cellular abnormalities. Electron microscopy showed that most THC2 platelets and megakaryocytes contain large amounts of Particulate Cytoplasmic Structures (PaCSs), a new cell component with selective immunoreactivity for polyubiquitinated proteins and protea-

some that have been recently identified in a number of solid cancers, in the epithelia of H. Pylori gastritis and related preneoplastic lesions, as well as in the neutrophils of Schwachman-Diamond Syndrome, a genetic disease with increased leukemia risk (Noris P et al. Blood 2011;117:6673-80).
PRELIMINARY CONCLUSIONS
Mutations in the 5'UTR of ANKRD26 identified in THC2 affect megakaryopoiesis and result in PaCS formation, thrombocytopenia and predisposition to acute leukemia. Since PaCS are typical of several malignant and pre-malignant conditions, the study of the functional consequences of ANKRD26 mutations can help to better understand not only the mechanisms of platelet production, but also those of oncogenesis.

ABSTRACT N. 191

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**LA VOLPE ADRIANA**	
Telethon grant N.	**GGP11076**	
Total budget €	**457.800**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2012**

NEW PHARMACOLOGICAL TARGETS IN FANCONI ANEMIA

Adamo Adele (1), Germoglio Marcello (1), Valenti Anna (2), Vettone Antonella (2), Perugino Giuseppe (2), Ciaramella Maria (2), Bottega Roberta (3), Comar Manola (3), De Rocco Daniela (3), Savoia Anna (3), La Volpe Adriana (1)

(1) CNR Institute of Genetics and Biophysics "A. Buzzati-Traverso", Via Pietro Castellino, 80131 Naples, Italy, Tel. 081.6132366, Fax 081.6132706, E-mail: adriana.lavolpe@igb.cnr.it
(2) CNR Institute of Protein Biochemistry, Naples, Italy
(3) IRCCS Burlo Garofolo, University of Trieste, Trieste, Italy

Fanconi anemia (FA) is a recessive disease caused by at least 15 different genes involved in DNA repair pathways. FA is characterized by progressive aplastic anaemia, multiple congenital abnormalities and high risk of developing hematological and solid tumors. The FA pathway branches to intersect with cell cycle control and apoptosis. Genes in the pathway are evolutionary conserved, allowing dissection and mechanistic studies in model systems, such as the nematode C. elegans. We previously demonstrated that inactivation of the Non-Homologous End Joining (NHEJ) pathway suppresses genomic instability due to defects of the FANCD2 gene in C. elegans and human cells (Adamo et al., 2010).
The FANCD2 protein, together with FANCI, is central to the FA pathway: activation of the pathway by DNA damage culminates with recruitment of ubiquitylated FANCD2-I complex to chromatin foci; however, the exact role of these proteins in the repair process in not understood.
3D models of the C. elegans FANCD2 and FANCI proteins, obtained using the 3D structure of the corresponding mouse complex, showed significant structural conservation. In order to dissect the biochemical activities of the FANCD2-I complex and identify its protein partners, we have undertaken the cloning, heterologous expression and purification of the C. elegans FANCD2 and FANCI proteins, as well as mutational analysis.
The checkpoint response to DNA damage is actively silenced in early C. elegans embryos. The one-cell embryo divides asymmetrically to produce the P1 and the AB daughter cells. The next round of cell division is asynchronous. Preliminary data showed that the time of division of the P1 cell is delayed after treatment with cisplatin in FANCD2 mutant (but not in other DNA repair mutants). This delay is reversed after depletion of lig-4 in fcd-2 mutant, suggesting that NHEJ is inappropriately activated in early fcd-2 embryos and is the cause of cell division delay and likely of associated developmental defects. This finding may explain the occurrence of congenital abnormality in FA patients. Analysis of a novel mutant suppressing the C. elegans fcd-2 phenotypes has been undertaken.
The polyomavirus SV40, a DNA tumor virus which spread into the human population through contaminated polio-vaccines, efficiently transforms FA fibroblasts suggesting that FA patients could be highly susceptible to SV40 infections. We detected the SV40 T-Ag in 25% of the FA patients. The SV40 positive patients were mainly males, all Italian, and most of them born before 1990. Due to a defective DNA repair pathway in FA, the SV40 virus is likely to persist longer in FA individuals. Further investigations in larger cohorts of patients are required to understand consequences of SV40 infection on progression and outcome of the disease.

ABSTRACT N. 192

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**PIETRANGELO ANTONELLO**	
Telethon grant N.	**GGP10233**	
Total budget €	**306.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

NOVEL STRATEGIES TO CURE HEREDITARY HEMOCHROMATOSIS THROUGH MODULATION OF THE BMP/SMAD PATHWAY REGULATING THE IRON HORMONE HEPCIDIN

Vecchi Chiara, Sabelli Manuela, Montosi Giuliana, Garuti Cinzia, Corradini Elena, Pietrangelo Antonello

Centro di Ricerca Epatologica Avanzata "Mario Coppo" (CREA), Dipartimento Scienze Mediche e Chirurgiche, Azienda Ospedaliero-Universitaria Policlinico di Modena, Via del Pozzo 71, 41100 Modena, Tel. 059.4222714; E-mail: antonello.pietrangelo@unimore.it

Background and rationale: Hereditary hemochromatosis (HH) is a disorder of iron metabolism characterized by progressive iron overload with potential for multi-organ damage and disease (Pietrangelo, N Engl J Med 350: 2383-2397, 2004). If untreated, HH can lead to liver cirrhosis, diabetes, cardiomyopathy, hypogonadic hypogonadism and arthritis. Phlebotomy is the standard treatment of HH. However this approach has limitations. Therefore new tools to remove/prevent iron excess as replacement/adjuvant of phlebotomy are highly anticipated.
Overall objectives: To develop novel strategies for the cure of HH by rescuing the defective expression of the iron hormone hepcidin. Specific aims of the first two years of the project have been: 1) To dissect the signals that control hepcidin transcription through the bone morphogenetic proteins (BMP) in mouse hemochromatosis; 2) to study biology and role of BMPs in human HH.
Results and Discussion. To determine if serum or hepatic iron (or both) regulates expression of Bmp6, the main hepcidin regulator, mice were fed an iron enriched diet for 1-5 weeks to obtain animals with different serum transferrin saturation (TS) and liver iron content (LIC). We found that both TS and LIC independently influence hepcidin expression. However, while TS activates the downstream BMP Smad1/5/8 signaling cascade, but does not induce Bmp6 mRNA expression, LIC independently correlated with hepatic Bmp6 mRNA expression and overall activation of the Smad1/5/8 signaling pathway. To study biology and role of BMPs, particularly BMP6, in HH, we preliminary used primary mouse hepatocytes exposed to low doses of exogenous a We found that all BMPs even at low doses were able to induce hepcidin mRNA and activate SMADs in WT hepatocytes. However, only BMP6 failed to induce hepcidin expression in Hfe knock out hepatocytes, indicating a specific involvement of HFE in the BMP6 post receptor signaling. Then, to study whether BMP6 is the BMP preferentially upregulated by iron also in humans and whether the BMP pathway is compromised in human HH, we analyzed liver biopsies from patients with iron overload due to HH or to non-HH. We found that also in humans liver BMP6 expression and SMAD signaling respond to iron burden. This occurs also in patients with HH. However, the correlation between iron excess and extent of BMP/SMAD signaling induction is lost in patients with HFE-HH, and maintained in subjects with non-hemochromatotic iron overload.
Overall the data data indicate that in human HFE-HH disrupted BMP/SMAD signaling in the liver is key in the pathogenesis of the disease and that BMP6 is a central player in this phenomenon. During the third year we will use exogenous BMPs to prevent, delay or reverse the iron overload phenotype in a Hfe-KO mouse model of hemochromatosis.

ABSTRACT N. 193

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**CAMASCHELLA CLARA**	
Telethon grant N.	**GGP12025**	
Total budget €	**349.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

HEMOCHROMATOSIS: FROM GENES TO CLINICS AND BACK

Camaschella Clara, Nai Antonella, Pagani Alessia, Rausa Marco, Silvestri Laura

Università Vita-Salute San Raffaele and San Raffaele Scientific Institute, Via Ol-

gettina, 60 20132 Milano, Italy, Tel. +39.0.26437782, Fax +39.02.26432640, E-mail: camaschella.clara@hsr.it

All types of hemochromatosis are due to insufficient production of the iron hormone hepcidin. This in juvenile forms results from mutations of hepcidin or of the BMP coreceptor hemojuvelin. However, in HFE- and TfR2-hemochromatosis, the cause of hepcidin underproduction remains unexplained. Also unclear are the mechanisms of hepcidin suppression in conditions of increased erythroid requests. Studies of IRIDA, an iron deficiency anemia with high hepcidin, the genetic condition opposite to hemochromatosis, and of its model Tmprss6-/- mouse, led us to propose that both TfR2 and HFE diseases mimic a condition of increased erythroid requests and are due to excessive activity of the serine protease TMPRSS6. We aim at demonstrating that TfR2 antagonizes this effect in cell and animal models. To define the relative position of Tmprss6 and Tfr2 we are crossing Tfr2- and Tmprss6-deficient mice. The preliminary studies on double knock out mice show that genetic loss of TMPRSS6 improves the phenotye of TFR2 hemochromatosis, modifying the mouse erythropoiesis. To explore TfR2 erythroid function we are selectively inactivating Tfr2 in the bone marrow in mice models for collaboration to evaluate the potential erythroid release and function of a soluble form of TfR2. To define the HFE function upstream TMPRSS6 we will explore whether it controls the iron carrier transferrin (or TMPRSS6) transcription/activity. We are also testing several inhibitors of TMPRSS6 in vitro and we are studying the mechanism of hemojuvelin cleavage by TMPRSS6 through a mutagenesis of the potential cleavage sites.
This project stems from our finding under the previous project GGP08089 that genetic loss of TMPRSS6 leads to hepcidin increase in conditions of high erythropoiesis in mice, aims to define the pathway of the so called erythroid regulator in hepcidin inhibition and its relationship with Tfr2 and to explore the pathogenesis of HFE-hemochromatosis. The preclinical studies aimed at impairing Tmprss6 activity are of potential applicability not only for the treatment of hemochromatosis but for all conditions of inappropriate activity of the erythroid regulator, as beta-thalassemia.

ABSTRACT N. 194

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GATTORNO MARCO**	
Telethon grant N.	**GGP09127**	
Total budget €	**302.600**	
Centres: **4**	*Duration (yrs):* **2**	*Starting year:* **2009**

EFFECTS OF CIAS-1 MUTATIONS IN THE PATHOGENESIS OF CRYOPYRIN ASSOCIATED PERIODIC SYNDROMES (CAPS). SEARCH FOR NEW GENES AND NOVEL THERAPEUTIC STRATEGIES

Gattorno Marco (1), Ceccherini Isabella (2), Giannoni Paolo (3), Rubartelli Anna (4)

(1) UO Pediatria 2, Istituto G. Gaslini, Largo G. Gaslini 5, 16147, Genova Tel. 010-5636793, E-mail: marcogattorno@ospedale-gaslini.ge.it
(2) Laboratorio di Genetica Molecolare, Istituto G Gaslini
(3) Centro di Biotecnologie Avanzate, Genova
(4) Istituto Nazionale per la Ricerca sul Cancro, Genova

1. We preliminarily showed that a condition of redox distress occurs in monocytes expressing mutated inflammasome genes, possibly affecting IL-1b processing and secretion. To investigate this point, we studied the redox state of resting monocytes from CAPS pts, the redox response to LPS, and the relationship between redox signaling and IL-1b secretion. As disease control, monocytes from pts with non-monogenic systemic idiopathic arthritis (JIA) were also studied.
LPS-induced IL-1b secretion is significantly accelerated in pts carrying CIAS1 mutations compared to healthy controls and SoJIA pts. The rapid secretion of IL-1ß is related to abnormal redox conditions of resting mutated monocytes and their consequent aberrant redox response to LPS. Redox distress in CAPS pts induces stress responses including protein synthesis inhibition with strong impairment of production of cytokines downstream of IL-1, such as IL-1Ra and IL-6. The deficient secretion of these cytokines coupled with increased and accelerated IL-1ß release explains the severity of the IL-1-related clinical manifestations in CAPS.
2. Generation of CIAS1-knock-in mice (in collaboration with Telethon facility). The murine NLRP3 mutation N475K (corresponding to the N477K human mutation associated to CINCA syndrome) was used. Following confirmation of germ-line transmission, chimeric CAPS mice has been provided by the facility. The chimeric mice with the highest content of mutant cells has been selected and mate to wild type (C57BL/6) to generate offspring heterozygous for N475K. Analysis of germline transmission has been performed by PCR. Heterozygous mice have been intercrossed to generate homozygous knock-in mice. Currently, we are breding these mice to various lines expressing Cre recombinase under control of different promoters.
3. A candidate gene approach has been applied to 7 CAPS-like pts negative for CIAS1 mutations. The following genes have been tested: NLRP12, CASP1, IL1RN, and IRAK1BP1. One pt with FCAS was positive for missense mutation of NLRP12. The other 6 CAPS-like pts were negative for the others genes. An exome sequencing approach has been undertaken in 2 pts in the Genomnia labs, after exclusion of somatic mosaicism. 14742 and 13428 single nucleotide substitutions were detected in the DNA of 2 samples, respectively. Of these, 386 and 374 are unreported in the dbSNP and in in-house exome sequencing analysis. After checking 17 genes commonly involved in variants found in both patients, and verifying that all the 34 variants under study had been inherited from one of the parents we are testing the following hypothesis: i) recessive inheritance of homozygous variants or the compound heterozygous condition for different genes in the 2 pts, ii) presence of severe mutations affecting a same pathway by assessing the degree of severity of all the >360 new mutations and their positioning in terms of which pathways the corresponding gene is involved in.

ABSTRACT N. 195

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**DI VIRGILIO FRANCESCO**	
Telethon grant N.	**GGP11014**	
Total budget €	**190.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE P2X7 RECEPTOR/ADENOSINE-GENERATION SYSTEM: A NOVEL TARGET FOR THE THERAPY OF AUTOINFLAMMATORY DISEASES

Sarti Alba Chiara, Falzoni Simonetta, Donvito Giovanna, Di Virgilio Francesco

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, via Borsari 46, 44121, Ferrara, Tel. 0532.455353, Fax .55351, E-mail: fdv@unife.it

Autoinflammatory diseases are a group of disorders characterized by episodes of "seemingly unprovoked" inflammation in the absence of auto-antibodies or antigen-specific T lymphocytes. Some of these disorders follow mendelian inheritance, while others show complex genetics, indicating interaction of multiple genetic loci. Some forms are also named "intrinsic inflammasomopathies" as the genetic defect is traced to the NLRP3 protein, the main constituent of the NLRP3 inflammasome. However the inflammasome is also central to the pathogenesis of autoinflammatory diseases where the mutated gene encodes extrinsic proteins with regulatory activity on the inflammasome (e.g. familiar mediterranean fever, FMF). A dysfunctional inflammasome is also central in the pathogenesis of Mevalonate kinase deficiency (MKD), or in the causation of autoinflammatory diseases with complex inheritance, such as the synovitis acne pustulosis hyperostosis osteitis (SAPHO) syndrome.
The common link between intrinsic and extrinsic inflammasomopathies is dysregulated IL-1beta secretion. Excessive IL-1beta secretion depends on gain-of-function mutations of NLRP3 (e.g. Muckle-Wells or familial cold autoinflammatory, FCAS, syndrome), loss-of-function mutation of pyrin (e.g. FMF), or dysregulated Akt activity (MKD). The NLRP3 inflammasome is activated by factors that bind to leucine-rich repeats (LRR) in the NALP3 molecule, by extracellular ligands of the purinergic P2X7 receptor (e.g. extracellular ATP), or by reactive oxygen species (ROS). So far, P2X7 is the most potent inflammasome activator among plasma membrane receptors, and its pharmacological blockade is a very efficient means to inhibit IL-1beta secretion. Furthermore, P2X7 is a main pathway for release of ATP, and thus for the generation of the potent anti inflammatory agent adenosine. In the initial phase of this project we are investigating (a) the role of extracellular ATP as a DAMP (damage-associated molecular pattern) and a trigger of "seemingly unprovoked" inflammation; (b) the role of P2X7 as a sensor for PAMPs (pathogen-associated molecular patterns) and DAMPs. Central to this investigation is the validation of novel probes that allow in vivo measurement of the extracellular ATP concentration. We are confident that a thorough understanding of the P2X7/ATP/adenosine

pathway will lead to novel and more efficient treatments for familial autoinflammatory diseases.

ABSTRACT N. 196

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FLEISCHHAUER KATHARINA**	
Telethon grant N.	**GGP08201**	
Total budget €	**291.700**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2008**

NEW IMMUNOGENETIC PARAMETERS FOR IMPROVING THE CURE OF INBORN GENETIC DISEASES BY ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Andreani Marco (2), Carcassi Carlo (3), Fleischhauer Katharina (1)

(1) Istituto Scientifico San Raffaele, via Olgettina 58, 20132 Milano, Tel. 02.2643.4341/6198, E-mail: fleischhauer.katharina@hsr.it
(2) Istituto Mediterraneo di Ematologia (IME), Roma
(3) Istituto Policlinico Universitario "Mario Aresu", Cagliari

Background: For many life-threatening genetic disorders, stable transfer of blood stem cells carrying the gene missing or defective in the patient by bone marrow transplantation (BMT) from healthy family or volunteer unrelated donors, is the only possible cure. The clinical success of this powerful therapeutic tool is however hampered by the immune response to transplanted cells, which is regulated by a number of genes with inter-individual sequence variability, called polymorphic genes. The emerging scenario suggests that each individual has a unique immunological risk profile determined by genetic imprinting. In this project, three Italian Centers of excellence from Milan, Rome and Cagliari, respectively, have joined their efforts to characterize a panel of polymorphic genes involved in the immune response to BMT for beta-thalassemia, a frequent inherited genetic disorder affecting the red blood system, and to study the function of specific immune cells in promoting or preventing adverse responses to BMT.
Results: Significant associations between adverse clinical outcome of BMT from family or unrelated donors were observed for a three different polymorphic gene systems, called HLA-G, KIR and CTLA-4, respectively. In addition, functional studies showed, for the first time, a hierarchy of immune responses to different targets, as well as a correlation of immunological tolerance with defined cellular subsets. These findings have important implications for donor selection, peri-transplant immune suppression and cellular therapies, and have led to 12 scientific publications in highly ranked peer-reviewed international journals.
Perspectives: The results from this project have direct implications for clinical risk prediction of the immune response not only in the context of BMT but also of other clinical settings such as gene therapy of inherited diseases, solid organ transplantation, autoimmunity and cancer.

ABSTRACT N. 197

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**CICARDI MARCO**	
Telethon grant N.	**GGP08223**	
Total budget €	**411.500**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2008**

GENE REGULATION IN THE PATHOGENESIS OF ANGIOEDEMA DUE TO INHERITED C1 INHIBITOR DEFICIENCY (HEREDITARY ANGIOEDEMA)

Caccia Sonia (2), Castellano Giuseppe (3), Zanichelli Andrea (1), Divella Chiara (3), Sallustio Fabio (7), Loreto Gesualdo (3), Bonanni Erika (1), Suffritti Chiara (1), Russo Rosaria (4), Bossi Fleur (5), Berardelli Romina (2,6), Fra Anna Maria (6), Rimoldi Valeria (2), Asselta Rosanna (2), Duga Stefano (2), Cicardi Marco (1)

(1) Dip. di Scienze Cliniche "Luigi Sacco", Università degli Studi di Milano, Ospedale Luigi Sacco, Via G.B. Grassi 74, 20157, Milano, Tel. +39.02.50319829, Fax +39.02.50319828, E-mail: Marco.Cicardi@unimi.it
(2) Dip. di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Milano
(3) Dip. di Emergenza e Trapianto di Organi, Università degli Studi di Bari, Bari
(4) Dip. di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Milano
(5) Dip. di Scienze della Vita, Università di Trieste, Trieste
(6) Dip. di Medicina Molecolare e Traslazionale, Università degli Studi di Brescia, Brescia
(7) Consorzio C. A. R. S. O., Valenzano, Bari

Hereditary Angioedema (HAE) is a rare Mendelian disease (estimated prevalence of 1:50,000) that causes recurrent local edema with important negative impact on the patients' quality of life and risk of death when the larynx is affected (Longhurst H, Cicardi M. Hereditary angio-oedema. The Lancet 2012; 379:474-481). Angioedema is mediated by bradykinin, released when a triggering factor, mainly physical and psychological stress, activates the contact system. Progress in the management of acute attacks has been done and several effective drugs are now available. However, knowledge and awareness for HAE remain very limited.
HAE is caused by mutations in the C1-inhibitor (C1-INH) gene. As an autosomal dominant disease, HAE patients are mostly heterozygous. Nevertheless, plasma levels of functional C1-INH in most HAE patients are markedly below the half normal levels that the wild-type allele should provide, suggesting a dominant-negative effect of the mutated allele on the wild-type one. To test this hypothesis, we combined total C1-INH mRNA measurement with allele-specific mRNA dosage in blood of HAE patients carrying different mutations. Generally, no difference in mRNA levels was found between controls and HAE patients carrying missense mutations, thus excluding a down regulation of the wild-type allele. Nonsense mutations were responsible for a marked allele-specific and position-dependent reduction of C1-INH transcript level, suggesting proper activation of the Nonsense-Mediated mRNA Decay pathway. Moreover, we identified mutations pointing to the presence of an mRNA stability element in the last exon. Expression studies in selected cell lines are underway.
Together with the specific studies on C1-inh gene, we follow a high-throughput approach, with the goal of identifying genes that are involved in angioedema attacks and thus potentially reduce the threshold for their triggering.
Using Illumina microarrays, we determined the effects of angioedema attack on the gene expression profile of 8 HAE patients comparing samples collected during and outside the attack and also from 8 healthy subjects (HS).
The microarray experiments revealed several modulated genes that have a potential function during the attack. We identified and validated 10 genes that were induced more than 1.5-fold during attack. These include genes that are important for the regulation of the vascular tone.
Gene Set Enrichment Analysis identified 10 processes differentially regulated comparing attack phase to inter-attack periods. The most represented processes ($p<0.05$) resulted in: "response to external stimulus", "protein processing" and "negative regulation of biosynthetic process". Moreover, comparing HAE patients in inter-attack periods to HS, we found the "cell matrix adhesion", "cell substrate adhesion" and "g protein signaling coupled to camp nucleotide second messenger" gene sets.

ABSTRACT N. 198

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BALDARI COSIMA**	
Telethon grant N.	**GGP11021**	
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Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE INTRAFLAGELLAR TRANSPORT SYSTEM, A NOVEL REGULATOR OF IMMUNE SYNAPSE ASSEMBLY: FUNCTIONAL DISSECTION IN T-CELL ANTIGEN RECEPTOR TRAFFICKING AND ASSESSMENT AS DISEASE TARGET IN COMMON VARIABLE IMMUNODEFICIENCY

Finetti Francesca (1), Patrussi Laura (1), Masi Giulia (1), Lucherini Orso Maria (1), Onnis Anna (1), Pazour Gregory J (2), Baldari Cosima (1)

(1) Dipartimento di Scienze della Vita, Università degli Studi di Siena, Via Aldo Moro 2, 53100 Siena, Tel 0577.234400; Fax 0577.234476; E-mail: baldari@unisi.it
(2) Program in Molecular Medicine, University of Massachussetts Medical School, Worcester, MA 01605, USA

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency, accounting for approximately 50% of inherited antibody deficiencies. The genetic aetiology has been established for only 15-20% of cases with the identification of mutations in three genes encoding direct (TACI, BAFF-R, CD19) or indirect

(ICOS) regulators of B-cell maturation. These findings have paved the way to the search of the genetic lesion in the large proportion of CVID of unknown aetiology, as they have identified lymphocyte activation as a central disease target. The T-cell antigen receptor (TCR) signaling defects found in a number of CVID patients pinpoint the TCR signaling machinery and its orchestration at the immune synapse (IS) between T cell and cognate APC as an attractive candidate for the search of the genetic defect in CVID cases of unknown aetiology.
We have recently demonstrated that the intraflagellar transport (IFT) system, which had been studied only in relation to its role in ciliogenesis, acts as a promoter of IS assembly in T cells by regulating polarized recycling of the TCR to this location, highlighting IFT is as a potential novel player in the membrane trafficking events required for T-cell activation. Here we have investigated the interplay of IFT20 with the Rab network that orchestrates receptor recycling. We show that IFT20 colocalizes and cofractionates with Rab5, which is associated with early endosomes, as well as with Rab4 and Rab11, which regulate the fast and slow recycling pathways, respectively. IFT20 knockdown results in a failure of Rab4 and Rab11 to polarize to the IS, indicating that it participates in both routes of receptor recycling. Consistent with this defect, analysis of the outcome of IFT20 knockdown on the TCR, as well as on the transferrin receptor (TfR) and the chemokine receptor CXCR4, all of which are recycling receptors, show that IFT20 is required for polarized recycling to the IS of both TCR and TfR, but not CXCR4, indicating that IFT20 is implicated in sorting these receptors during their intracellular trafficking though the recycling compartment. We also show that all the components of the IFT system are expressed in T cells, and provide evidence that two other IFT polypeptides, IFT54 and IFT57, participate as essential players in the recycling pathways regulated by IFT20 in these cells.
Collectively, the results provide novel insights into the mechanisms that control polarized TCR recycling to the IS and underscore the interplay between the IFT system and the exocytic machinery in IS assembly. The results, which provide new candidates for the genetic classification of CVID of unknown aetiology, will be translated to the pathological setting of the T-cell activation defects in these patients.

ABSTRACT N. 199

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FERRARI SIMONA**	
Telethon grant N.	**GGP12052**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **1**	*Starting year:* **2012**

IDENTIFICATION OF THE GENE RESPONSIBLE FOR AN AUTOSOMAL DOMINANT FORM OF COMMON VARIABLE IMMUNODEFICIENCY

Di Pierro Valentina (1), Lyons Paul (2,3), Zuntini Roberta (1), Schaffer Alejandro (4), Quinti Isabella (5), Grimbacher Bodo (6), Smith Ken (2), Ferrari Simona (1)

(1) Laboratory of Medical Genetics, S.Orsola-Malpighi-University Hospital, via Massarenti 9, 40138, Bologna, Italy, Tel. +39.051.2082410, Fax +39.051.2082416, E-mail: simo.ferrari@unibo.it
(2) Cambridge Insitute of Medical Research, University of Cambridge, UK
(3) Wellcome Trust Sanger Institute, Hinxton, UK
(4) National Center for Biotechnology Information, NIH, Bethesda, USA
(5) Dept of Immunology, University "La Sapienza", Roma, Italy
(6) Centre of Chronic Immunodeficiency, University Medical Centre Freiburg, Germany

CVID is the most common symptomatic antibody deficiency. It includes a heterogeneous group of disorders characterized by defects in the terminal stage of B lymphocyte differentiation, leading to markedly reduced immunoglobulin serum levels, poor response to vaccines and increased susceptibility to bacterial infections. The majority of CVID cases are sporadic. Approximately 20% are familial; rare autosomal recessive mutations in ICOS, BAFF-R, CD19, CD20, CD81 and CD21 coding genes have been recently reported and mutations in the TACI gene have been found in about 15% of cases. However, the underlying genetic defects remain unknown in the majority of cases.
Here we report a large family with autosomal dominant inheritance of CVID, diagnosed according to the ESID/PAGID criteria for CVID based on a marked decrease (at least 2 SD below the mean for age) in serum IgG and IgA, onset age >2 years, poor response to vaccines and exclusion of other causes of hypogammaglobulinemia.
In this five-generation family, nine affected and eight unaffected individuals were available for the study. In order to identify genetic variants responsible for CVID, genomewide linkage scan was performed. The CVID locus has been mapped to the long arm of chromosome 3 on band q27.2-q29. The presence of several recombination events localized the CVID gene in a region of 9.2 Mb spanning from marker D3S3570 to marker D3S1265, with a maximum LOD score of 3.90 at ?=0.00 for marker D3S2747.
In collaboration with the Wellcome Trust Sanger Institute, we performed Exome sequencing in four affected individuals, in order to identify shared rare variants within the linkage region. We are in the process of analyzing the Exome sequencing data.
Reports of families with autosomal dominant CVID in several generations are extremely rare. The family reported in this study provides a unique opportunity for seeking a new gene responsible for CVID.

ABSTRACT N. 200

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GRAZIANI ANDREA**	
Telethon grant N.	**GGP10034**	
Total budget €	**193.600**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

ROLE OF DIACYLGLYCEROL KINASE ALPHA IN SAP FUNCTION: IMPLICATIONS FOR T-CELL MEDIATED IMMUNE RESPONSE IN X-LINKED LYMPHOPROLIFERATIVE DISEASE (XLP) PATIENTS AND AS POSSIBLE PHARMACOLOGICAL TARGET FOR XLP TREATMENT

Baldanzi Gianluca (1), Ruffo Elisa (1), Pighini Andrea (1), Patrussi Laura (2), Baldari Tatiana (2), Nichols Kim (3), Snow Andrew (4), Graziani Andrea (1)

Dept. of Translational Medicine, University of Piemonte Orientale, Via Solaroli 17, 28100, Novara, Tel. 0321-660619, E-mail: graziani@med.unipmn.it
(2) Dept. of Evolutionary Biology, University of Siena, Italy
(3) Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, PA, USA
(4) Dept of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

X-linked lymphoproliferative disease (XLP) is a rare congenital immunodeficiency, caused by loss of expression of SLAM-associated protein (SAP), an SH2-containing protein adaptor, which is essential for SLAM-mediated signaling and contributes to TCR/CD28-induced signaling and T cell activation. SAP deficiency leads to an extreme, usually fatal increase in the number of lymphocytes upon infection with EBV. Recent evidence showed that SAP deficiency causes resistance to apoptosis mediated by TCR restimulation-induced cell death (RICD), a process that normally constrains T cell expansion during immune responses. However the mechanisms by which SAP transduces TCR restimulation cell death are not known.
We recently showed that upon TCR stimulation, SAP mediates negative regulation of the enzymatic activity of Diacylglycerol kinase alpha (DGK-alpha) (Baldanzi et al. J. Immunol. 2011). DGK-alpha is an enzyme that, by phosphorylating diacylglcyerol to phosphatidic acid, acts as a negative regulator of TCR signaling and induces T cell anergy. Upon co-stimulation of the TCR with CD28 or SLAM, DGK-alpha, but not DGK-zeta, exits from the nucleus and undergoes rapid negative regulation of its enzymatic activity in a SAP-dependent manner. Moreover, over-expression of SAP is sufficient to inhibit DGK-alpha, while SAP mutants unable to bind either phospho-tyrosine residues or SH3 domain are ineffective. In SAP-deficient Jurkat cells inhibition of DGK-alpha partially rescues defective TCR/CD28 signaling, including Ras and ERK-1/2 activation, PKC-theta membrane recruitment, induction of NF-AT transcriptional activity and IL-2 production.
Finally, we set to investigate whether RICD may be induced through SAP-dependent negative regulation of DGK-alpha. Intriguingly, either DGK-alpha silencing or pharmacological inhibition efficiently restore RICD in SAP deficient Jurkat cells, as well as T cells from both XLP patients and SAP KO mice. Our data indicate that SAP-mediated inhibition of DGK-alpha sustains diacylglycerol signaling, thereby enhancing T cell activation and RICD. Those data also suggest DGK-alpha inhibition as a novel pharmacological strategy for XLP treatment.

ABSTRACT N. 201

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**VIOLA ANTONELLA**	
Telethon grant N.	**GGP10170**	
Total budget €	**284.800**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2011**

UNDERSTANDING THE WHIM SYNDROME: MOLECULAR ANALYSIS OF CXCR4 FUNCTIONS IN LEUKOCYTE TRAFFICKING AND ACTIVATION

Kallikourdis Marinos (1,3), Trovato Anna Elisa (1), Anselmi Fabio (1), Sarukhan Adelaida (1), Tassone Laura (2), Badolato Raffaele (2), Viola Antonella (1,3)

(1) Laboratorio Immunità Adattativa, Fondazione Humanitas per la Ricerca, Via Manzoni 56, Rozzano (MI), Italy, Tel. 02.82245118. Fax 02.82245101, E-mail: antonella.viola@humanitasresearch.it
(2) Clinica Pediatrica, University of Brescia, c/o Spedali Civili, Brescia, Italy
(3) Dipartimento BioMeTra, Università degli Studi di Milano

The WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) syndrome is a rare human disease characterized by neutropenia, myelokathexis, delayed antibody class switching, hypogammaglobulinemia, recurring infections, impaired memory B cell function and human papillomavirus (HPV)-induced warts. It is caused by C-terminal truncating, dominant mutations in the chemokine receptor CXCR4. This has been shown to impair the intracellular trafficking of the receptor in neutrophils, leading to increased responsiveness to chemokine and retention of neutrophils in the bone marrow, thus explaining the myelokathexis and neutropenia. Yet the extensive defects in the adaptive immunity of WHIM patients remain largely unexplained. We hypothesized that the WHIM-associated mutations in CXCR4 may affect the formation of immunological synapses between T cells and antigen presenting cells (APC). We show that WHIM-mutant CXCR4 disrupts the stability of T cell-APC conjugates in WHIM patients, in the presence of competing external chemokine ligand. This is due to an impairment of receptor recruitment to the T-APC synapse. Further, using a retrogenic mouse that develops WHIM-mutant T cells, we show in ex vivo lymph node slice 2-photon microscopy that WHIM-mutant CXCR4 inhibits the formation of T cell-APC long-lasting interactions and impairs in vivo T cell activation and its downstream effects. These findings demonstrate that chemokine receptors can affect the stability of the immunological synapse and allow us to propose a mechanism that contributes directly to the observed adaptive response-related symptoms of the WHIM syndrome.

ABSTRACT N. 202

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BENVENUTI FEDERICA**	
Telethon grant N.	**GGP10231**	
Total budget €	**121.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

A LINK BETWEEN TOLL-LIKE RECEPTOR SIGNALING IN DENDRITIC CELLS AND AUTOIMMUNE MANIFESTATIONS IN WISKOTT-ALDRICH SYNDROME

Prete Francesca (1), Catucci Marco (2), Labrada Mayrel (3), Gobessi Stefania (4), Castiello Maria Carmina (5), Bonomi Elisa (6), Aiuti Alessandro (7), Vermi William (6,8), Cancrini Caterina (9), Metin Ayse (10), Hambleton Sophie (11), Bredius Robbert (12), Notarangelo Luigi-Daniele (13), Van der Burg Myriam (14), Kalinke Ulrich (15), Villa Anna (2,16), Benvenuti Federica (1)

(1) Federica Benvenuti. Cellular Immunology, ICGEB, Area di Ricerca, Padriciano 99, Trieste, Italy, Tel. 040.3757389, Fax 040.226555
(2) San Raffaele Scientific Institute, Telethon Institute for Gene Therapy, Milan, Italy
(3) Immunobiology Division, Center of Molecular Immunology, Havana, Cuba
(4) International Centre for Genetic Engineering and Biotechnology (ICGEB) Molecular Hematology Group, Campus "A. Buzzati-Traverso," Rome, Italy
(5) Vita-Salute San Raffaele University, Milan, 20132, Italy
(6) Department of Pathology, University of Brescia/Spedali Civilli di Brescia, Brescia, Italy
(7) Department of Public Health and Cell Biology, Tor Vergata University, Rome, Italy
(8) Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110, USA
(9) Department of Pediatrics, Children's Hospital Bambino Gesù and Tor Vergata University, Rome, Italy
(10) Pediatric Immunology and Allergy Department, Ankara Children's Diseases. Hematology Oncology Training Hospital Pediatric Hematology, Bone Marrow, Transplantation Unit, Ankara Children's Hematology Oncology Training Hospital, Ankara, Turkey
(11) Institute of Cellular Medicine, Newcastle University, NE2 4HH, UK
(12) Department of Hematology and Immunology of Leiden University Medical Center, Leiden, The Netherlands
(13) Division of Immunology, Children's Hospital, Harvard Medical School, Boston, MA, USA
(14) Department of Immunology, Erasmus MC, University Medical Center Rotterdam, 3015 GE Rotterdam, The Netherland
(15) Institute for Experimental Infection Research, TWINCORE, Hanover, Germany
(16) Milan Unit, Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche, Milan, Italy

Mutations in Wiskott-Aldrich syndrome protein (WASp), a regulator of actin dynamics in hematopoietic cells, cause WAS an X-linked primary immunodeficiency characterized by recurrent infections and a marked predisposition to develop autoimmune disorders. The mechanisms that link actin alterations to the autoimmune phenotype are still poorly understood. Here we show that chronic activation of plasmacytoid dendritic cells (pDCs) and elevated type-I interferon levels play a role in WAS autoimmunity. WAS patients display increased expression of type-I interferon genes and their inducible targets, alteration in pDCs numbers and hyperresponsiveness to TLR9. Importantly, ablating IFN-I signaling in WASp null mice rescued chronic activation of conventional DCs, splenomegaly and colitis. Using WASp deficient mice we demonstrated that WASp null pDCs are intrinsically more responsive to multimeric agonist of TLR9, constitutively secrete type-I interferon but become progressively tolerant to further stimulation. By acute silencing of WASp and actin inhibitors we show that WASp-mediated actin polymerization controls intracellular trafficking and compartmentalization of TLR9 ligands in pDCs restraining exaggerated activation of the TLR9/ ?FN-a pathway. Together these data highlight the role of actin dynamics in pDCs innate functions and imply the pDCs/ ?FN-a axis as a player in the onset of autoimmune phenomena in WAS disease.

ABSTRACT N. 203

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MURO ANDRES FERNANDO**	
Telethon grant N.	**GGP10051**	
Total budget €	**435.900**	
Centres: **5**	*Duration (yrs):* **3**	*Starting year:* **2010**

LIFE-LONG LIVER SPECIFIC AAV-MEDIATED GENE THERAPY IN A CRIGLER-NAJJAR MOUSE MODEL

Bortolussi Giulia (1), Bockor Luka (1), Zentilin Lorena (1), Mancarella Antonio (1), Bellarosa Cristina (2), Giacca Mauro (1), Tiribelli Claudio (2), Muro Andrés F. (1)

(1) International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano, 99, 34149, Trieste, Italy, Tel. 040.3757369, Fax 040.226555, E-mail: muro@icgeb.org
(2) Fondazione Italiana Fegato, Centro Studi Fegato, Trieste, Italy

Crigler-Najjar syndrome type I (CNSI) is a rare genetic disorder characterized by the inability to conjugate bilirubin due to uridine diphospho-glucuronosyl transferase 1a1 (UGT1a1) deficiency. As a consequence, babies have high levels of unconjugated bilirubin and are at constant risk of developing neurological damage unless phototherapy (PT) is applied since birth. However, during puberty PT becomes less effective, and liver transplantation is required.
To find alternative therapies to permanently correct the genetic defect we generate a mouse model of CNSI that closely reproduce all major features of CNSI such as neonatal hyperbilirubinemia, extensive neuronal cell death, motor impairment and ultimately death. We previously showed that neonatal gene transfer of the CMV-hUgt1a1 gene was able to rescue all mutant mice. As the therapeutic gene was expressed in skeletal muscle but not in the liver, to promote liver-specific expression we here used an AAV vector carrying the enhancer element of ApoE gene and the minimal promoter region of alpha1antitrypsin (AAT); the viral vector was packed in AAV serotype 8. Mutant mice were treated at P4 with a single injection of the therapeutic gene. All gene therapy-treated mice reached

adulthood without any apparent neurological damage. Bilirubin levels were 70-80% lower than untreated controls at 1 and 2 months after injection. At 17 months post-injection, injected mice showed a serum bilirubin level 50% lower compared to untreated controls and well below from the risky threshold of neurological damage. We compared the liver-specific gene therapy of AAV8-ATT-hUGT1a1 with that of the AAV9-CMV-hUGT1a1, previously shown to express the therapeutic gene in skeletal muscle. Although the drop in bilirubin levels was comparable between the 2 treatments, liver tissue expressed 60 times less hUGT1a1 mRNA than skeletal muscle. Western blot analysis revealed only 1% liver expression of hUGT1a1, as compared to wt. Interestingly this amount is sufficient to maintain lifelong low levels of plasma bilirubin. We speculate that this remarkable difference in the efficiency of the hUGT1a1 is related to the expression in liver, but not in muscle, of the Mrp2 transporter, which accounts for the biliary excretion of conjugated bilirubin.
In conclusion, we demonstrated that neonatal gene transfer of liver specific hUGT1a1 is able to rescue the lethal phenotype of mutant mice and extend their lifespan up to 2 years. Moreover, we showed that 1% of UGT1a1 expression levels is sufficient to reduce bilirubin levels in mutant mice well below from the risk of developing neurological damage. These data confirm that liver is the appropriate target organ for long-term CNSI gene therapy, ruling out the possibility of using extra-hepatic tissues such as skeletal muscle.

ABSTRACT N. 204

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GALIETTA LUIS**	
Telethon grant N.	**GGP10026**	
Total budget €	**391.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

IDENTIFICATION OF NOVEL STRATEGIES TO CORRECT THE CHLORIDE TRANSPORT DEFECT IN CYSTIC FIBROSIS

Pedemonte Nicoletta, Caci Emanuela, Sondo Elvira, Ferrera Loretta, Tomati Valeria, Scudieri Paolo, Zegarra-Moran Olga, Ravazzolo Roberto, Galietta Luis J.V.

Lab. di Genetica Molecolare, Ist. G. Gaslini, 16147 Genova, Tel. 010.5636801, Fax 010.3779797, E-mail: galietta@unige.it

Cystic fibrosis (CF) is caused by reduced anion transport in the epithelial cells of various organs. In the lungs, the basic defect causes bacterial infections and plugging of the airways by highly dense mucus secretions. Our general aim is to develop novel pharmacological strategies to correct the basic defect in CF. To this purpose, we are considering two different targets: CFTR, the plasma membrane chloride channel protein that is altered by CF mutations, and the calcium-activated chloride channel (CaCC), which represents an alternative pathway to circumvent the basic defect. Our project addresses two specific aims: 1) the identification of novel targets and the development of small molecules to correct the molecular defect caused by F508del, the most frequent mutation affecting CF patients; 2) the functional and molecular characterization of TMEM16A protein as a major component of the epithelial CaCC.
The F508del mutation causes impaired maturation of the CFTR protein. Indeed, mutant CFTR is detected as a misfolded protein during its biosynthesis and early tagged for degradation. Although a small fraction of F508del-CFTR reaches the plasma membrane, it is rapidly internalized and degraded. To rescue F508del-CFTR, we have screened a genome-wide library of short interfering RNAs (siRNA) using a high-throughput functional assay based on the halide-sensitive yellow fluorescent protein. The screening has identified a series of proteins, involved in ubiquitination and sumoylation, whose silencing leads to improved trafficking of the mutant protein to the plasma membrane. In parallel, we have identified small molecules, belonging to the class of aminoarylthiazoles, that cause a dual effect on F508del-CFTR. Such molecules improve both the trafficking and the channel gating of the mutant protein.
We are also studying the TMEM16A protein since it is a major component of epithelial CaCC. Interestingly, we have found that TMEM16A expression in the airway epithelium is particularly associated with mucus cell metaplasia, a condition of mucus hypersecretion that is typical of CF, asthma, and other chronic respiratory diseases. In particular, TMEM16A is highly expressed in goblet cells whereas CFTR is expressed in ciliated cells. Such results suggest that TMEM16A may be particularly required for mucin secretion. The separate localization of TMEM16A and CFTR requires further investigation to assess whether the former protein is a suitable drug target for CF therapy.

Publications:
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2. Pedemonte et al. (2011) J Biol Chem 286:15215-15226
3. Budriesi et al. (2011) J Med Chem 54: 3885-3394
4. Ferrera et al. (2011) Biochim Biophys Acta 1808: 2214-2223
5. Sondo et al. (2011) Am J Physiol 301: C872-C885
6. Scudieri et al. (2012) J Physiol (in press)

ABSTRACT N. 205

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MAIURI LUIGI**	
Telethon grant N.	**GGP12128**	
Total budget €	**293.700**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2012**

THE HEME-OXYGENASE 1 (HO-1) AS MODULATOR OF CYSTIC FIBROSIS LUNG DISEASE

Villella Valeria (1), Ferrari Eleonora (1), Esposito Speranza (1), Kyritsi Konstantina (1,2), Monzani Romina (1), Egan Marie (2,3), Maiuri Maria Chiara (4,5), Kroemer Guido (5), Raia Valeria (6), Bruscia Emanuela (1,2), Maiuri Luigi (1)

(1) European Institute for Research in Cystic Fibrosis (IERFC) by San Raffaele Hospital, Via Olgettina 60, 20132, Milan, Tel. +39.320.7981514, Fax 39.02.26434328, E-mail: maiuri.luigi@hsr.it
(2) Pediatrics, Yale University School of Medicine, New Haven, USA
(3) Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, USA
(4) Experimental Pharmacology, University Federico II, Naples, Italy
(5) INSERM U848 - Institut Gustave Roussy, Villejuif, France
(6) Pediatrics, University Federico II, Naples, Italy

In Cystic Fibrosis (CF) pathology, the tightly regulated relationship between oxidative stress and inflammation is unbalanced, as a consequence of defective CFTR function. This favors chronic lung inflammation, susceptibility to bacterial infection and colonization and tissue damage over time. The mechanism/s by which lack of functional CFTR leads to an unregulated control of oxidative stress and inflammation is unknown.
The heme-oxygenase 1 (HO-1)/carbon monoxide (CO) is a key cellular pathway that once induced can modulate the redox status of the cells, the inflammatory response, bacterial killing, and putatively autophagosome formation (Waltz, et al., 2011).
We have discovered that the HO-1/CO pathway is defective in CF and we hypothesize that this defect may be responsible for the unbalanced redox regulation together with decreased induction of cytoprotective pathways in CF. The overall goal of our study is to investigate the role of this pathway in CF airway epithelial cells, macrophages and in CF pre-clinical mice models. Here we show that in Pseudomonas aeruginosa (PA)-LPS treated CF macrophages, in which HO-1/CO acts as negative regulator of the Toll like receptor 4 (TLR4) signaling (Wang et al 2009), the HO-1 fails to compartmentalize to the cell surface, as we observed in WT cells, but accumulates intracellularly. Over-expressing HO-1 with adenoviral vector or by delivering CO with the CO releasing molecule enhances HO-1 expression and reestablishes co-localization with TLR4 on the CF cells surface. Accordingly, these treatments decreased the production of pro-inflammatory cytokines in LPS trigged CF macrophages. The HO-1 compartmentalization to the cell surface has been linked to the scaffold protein caveolin-1 (Cav-1) (Wang et al 2009). Therefore, we will test whether the HO-1 mislocation in CF macrophages is associated with defective Cav-1 expression. We are also evaluating whether this pathway may play a role in autophagy induction and whether its modulation ameliorates the defective bacterial killing observed in CF macrophages (Abdulrahman et al., 2011).
We also show that human CF bronchial epithelial cells lines (CFBE41o-) treated with LPS or PA had defective induction of the HO-1 when compared to WT bronchial epithelial cells (16HBE14o-) and that overexpression of HO-1 via genetic manipulations decreases oxidative stress and reduces inflammatory markers. We are evaluating whether targeting HO-1 might also rescue autophagy in CF epithelia.
Finally, we show that over-expression of HO-1 in CF mice decreases the lung hyper-inflammatory response to LPS, which characterizes these mice (Bruscia et al., 2009; Luciani et al. 2010).
We are now in the process of performing pre-clinical studies using FDA approved drugs that act stimulating endogenous HO-1 activity in in vitro and in vivo CF models. At the end of this study, we will understand whether modulation of this pathway can be considered a potential therapeutic intervention for CF.

ABSTRACT N. 206

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**STRAZZABOSCO MARIO**	
Telethon grant N.	**GGP12133**	
Total budget €	**152.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2013**

CFTR-DEFICIENCY CAUSES A DYSREGULATION IN TOLL-LIKE RECEPTOR-MEDIATED INNATE IMMUNE RESPONSES: PATHOGENETIC AND THERAPEUTIC IMPLICATIONS FOR CYSTIC FIBROSIS-RELATED LIVER DISEASE

Fiorotto Romina (2), Scirpo Roberto (1), Morell Carola Maria (1), Strazzabosco Mario (1)

(1) Department of Surgery and Interdisciplinary Medicine; University of Milano-Bicocca, Via Cadore 48, 20900 Monza, Italy, Tel. 02.64488052, Fax 02.64488260, E-mail: mario.strazzabosco@unimib.it
(2) Department of Internal Medicine; Yale University; New Haven (USA)

Background: CF-associated liver disease (CFLD) negatively impacts the quality of life and survival of CF patients. Consistent with the specific expression of CFTR in the biliary tree, CFLD is characterized by a chronic cholangiopathy that can eventually evolve into sclerosing cholangitis and focal biliary cirrhosis. Liver disease in patients with CF has been classically considered a consequence of the impaired bile flow and biliary alkalinization caused by the defective channel function of CFTR. However, the pathogenesis of CFLD is not well understood and the treatment is limited to the administration of choleretic bile acids.
Using an experimental model in which CFTR-defective biliary cells were exposed to endotoxins, we have recently found that cholestasis is not the primary event involved in the induction of the biliary damage and that a "two hit phenomenon" (i.e endotoxemia) is needed to develop the liver phenotype.
Hypothesis and objectives: Our hypothesis is that CFTR participates to the regulation of epithelial innate immunity, by controlling toll-like receptors (TLR) mediated responses. We aim to investigate the pathogenetic role of TLRs in CFLD, the mechanistic relationships between CFTR and TLR-mediated signaling, and novel therapeutic approaches based on these interactions.
Methods: We will study whether TLR2, 4 and 5 are regulated by CFTR in murine primary cholangiocytes cell lines, and use liver specific MyD88/CFTR double KO mice to demonstrate the pathogenetic role of TLRs in CFLD in vivo. Then, we will address the role of protein kinase Src as the mechanistic link between CFTR deficiency and TLR stimulation. We will study if blockage of Src or of TLR4 signaling prevents biliary damage in Cftr-KO mice. Finally, we will study the potential anti-inflammatory therapeutic value of PPAR gamma agonists in vitro and vivo.
Expected results: We expect to find that CFTR is involved in the regulation of epithelial innate immunity and that CFLD results from an altered TLRs-dependent immune response of the biliary epithelium. Also, we will prove the therapeutic benefit of interfering with several signaling proteins involved in epithelial innate responses.
Spin-off for research & clinical purposes: These studies will change our current understanding of the pathogenesis and treatment of CF-cholangiopathy. This novel interpretation of the pathogenesis of CFLD may have important therapeutic consequences and significantly impact on the management of CFLD.

ABSTRACT N. 207

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MILANI SILVANO**	
Telethon grant N.	**GGP12258**	
Total budget €	**66.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

IMPROVING THE DIAGNOSTIC ACCURACY OF A NEONATAL SCREENING PROTOCOL FOR CYSTIC FIBROSIS: CHOICE OF THE OPTIMAL THRESHOLDS FOR THE BLOOD LEVEL OF IMMUNOREACTIVE TRYPSIN (IRT)

Milani Silvano (1), Bossi Anna (1), Corbetta Carlo (2)

(1) Department of Clinical Sciences and Community Health, Unit of Medical Statistics and Biometry, University of Milan, via Venezian 1, 20133 Milano, Italy, Tel. 02.503.20854, Fax 02.503.20866, E-mail: silvano.milani@unimi.it
(2) Laboratorio di Riferimento Regionale per lo Screening Neonatale, Ospedale dei Bambini "Vittore Buzzi", AO Istituti Clinici di Perfezionamento, Milan, Italy

Background. Cystic Fibrosis (CF) is an inheritable autosomal recessive disease that causes chronic pulmonary infections, gastrointestinal and nutritional abnormalities, salt loss syndromes, and congenital bilateral absence of the vas deferens in males. The early diagnosis and treatment were demonstrated to improve patients' quality of life and disease's prognosis. In this context, neonatal screening plays a crucial role, provided that its diagnostic accuracy is very high. Actually, false negative results imply delayed diagnosis and therapy, whereas false positive results induce undue anxiety to the families of healthy babies. In Lombardy, at present, the ratio of false to true positive results is 60:1.
Aims. This research programme aims at assessing the performance of possible strategies for the neonatal screening of Cystic Fibrosis, in terms of sensitivity, specificity and cost-effectiveness ratio.
Design and methods. Among the factors that should be considered to improve the diagnostic accuracy of a screening programme for CF, this research will focus on methods suitable to choose optimal thresholds for the blood level of immunoreactive trypsin (IRT) measured on dried-blood spot specimens. The threshold dependent sensitivity and specificity will be estimated on the basis of data provided by the "Laboratorio di Riferimento Regionale per lo Screening Neonatale dell'A.O. Istituti Clinici di Perfezionamento (ICP)" of Milano, which performs neonatal screening of all babies born in Lombardy (about 100,000 per year). A database structure will be designed and implemented to store the screening data (about 30 items per neonate) of all babies screened between 2004 and 2012 (about 220 CF patients expected out of 850,000 neonates). The list of all CF patients positive on the screening will be cross-checked with the list of all CF patients, so as to estimate of the number of false negative results. The median time period of the study (about 4.5 years) is long enough to detect almost all children with CF who were missed on screening. The distribution of IRT values derived from routine screening will be used to evaluate the effect of varying the thresholds on sensitivity and specificity. At present, the IRT threshold in use to detect CF neonates is 70 ng/ml, corresponding to the 99th centile of the IRT distribution in the reference population, i.e. the threshold that, in the reference population, ensures a 99% specificity and 1% of false positive results.
Anticipated output. The definition of a rational strategy to choose optimal thresholds could save parents the trouble, anxiety and discomfort due to a false positive result, while contributing to reduce costs for the National Health Service in terms of IRT, DNA and sweat tests not performed. The new thresholds could be first adopted in Lombardy and then in other Italian Regions, if appropriate.

ABSTRACT N. 208

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**VANONI MARIA ANTONIETTA**	
Telethon grant N.	**GGP10090**	
Total budget €	**206.800**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

STRUCTURE-FUNCTION STUDIES ON 24-DEHYDROCHOLESTEROL REDUCTASE, THE AFFECTED ENZYME IN DESMOSTEROLOSIS, A SEVERE INHERITED DISORDER OF STEROL METABOLISM

Zucchini Daniela (1), Zito Arianna (2), Cuevas Polo Nerea (3), Galbiati M. Rita (2), Caruso Donatella (2), Maggi Roberto (2), Tedeschi Gabriella (3), Vanoni Maria Antonietta (1)

(1) Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano Italia, Tel. +39.02.50314901; Fax +39.02.50314895; E-mail: maria.vanoni@unimi.it
(2) Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milano
(3) Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, Milano

Desmosterolosis is a severe, although rare, inherited disease, which causes marked developmental defects and is associated with mutations of the gene encoding the 24-dehydrocholesterol reductase (DHCR24). The corresponding enzymatic activity has been detected over 20 years ago, but the enzyme has not been isolated yet. Thus, its actual catalytic properties and the correlation among its role in cholesterol biosynthesis, the recently reported antiapoptotic function and of protection from oxidative stress, and the observed serious developmental defects are not known. For these reasons, this

project aims (i) to produce and isolate the DHCR24 enzyme to carry out its biochemical characterization in vitro, and (ii) to study its role in cell lines of neuronal origin.
Analyses of DHCR24 primary structure, as predicted from the available cDNAs sequences, and of its subcellular localization, based also on our own experiments, indicate that DHCR24 is a flavoprotein formed by an N-terminal (ca. 50 residues) region anchoring it to the endoplasmic reticulum membrane followed by a soluble catalytic domain. Thus, we have engineered the human DHCR24 cDNA for the overproduction of soluble and catalytically active protein forms in E.coli and S.cerevisiae. As a result of several expression experiments, we have eventually obtained soluble forms of DHCR24 lacking the putative N-terminal membrane-anchoring region, which are, however, devoid of the flavin cofactor and aggregated, to indicate (partial) misfolding. These forms can be purified to homogeneity in the presence of denaturants or detergents opening the way to refolding experiments and to the production of novel anti-DHCR24 antibodies. In this respect, it is worth mentioning that the commercially available antiDHCR24 antibodies have been found not to be suitable for experiments designed to locate endogenous DHCR24 forms in tissues and cells in that they recognize several proteins, none of which corresponding to DHCR24 forms, as shown by proteomic approaches.
To understand the DHCR24 role in cells, the corresponding cDNA has been re-engineered to express (transiently or stably, for greater experimental reproducibility) full-length and N-terminally truncated DHCR24 forms in Gn11 cell lines, These cells have been selected because they express low levels of endogenous DHCR24. For these experiments DHCR24 is produced in fusion with GFP (as done so far by several authors) or with a FLAG peptide. The small size of the FLAG peptide, as compared with GFP, should avoid artifacts due to direct interference of the tag with DHCR24 properties. With these constructs we have developed methods to correlate levels and subcellular location of expressed DHCR24 forms (cytochemical and biochemical methods), levels of cholesterol precursors and derivatives, including those playing a regulatory role (by gas chromatography and mass spectrometry) and the cells response to oxidative stress.

ABSTRACT N. 209

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**VOLTATTORNI CARLA**	
Telethon grant N.	**GGP10092**	
Total budget €	**213.200**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2010**

DEVELOPMENT OF NEW STRATEGIES FOR THE TREATMENT OF PRIMARY HYPEROXALURIA TYPE I

Cellini Barbara (1), Montioli Riccardo (1), Oppici Elisa (1), Roncador Alessandro (1), Bianconi Silvia (1), Amoroso Antonio (5), De Marchi Mario (2), Mandrile Giorgia (2), Peruzzi Licia (4), Marangella Martino (3), Voltattorni Carla (1)

(1) Department of Life Sciences and Reproduction, Section of Biological Chemistry, University of Verona, Strada le Grazie 8, 37134 Verona, Tel 045.8027175, E-mail: carla.borrivoltattorni@univr.it
(2) Department of Clinical and Biological Sciences, University of Torino
(3) Nephrology Unit, Ospedale Mauriziano, Torino
(4) Pediatric Nephrology Unit, Children Hospital OIRM Sant'Anna, Torino
(5) Department of Genetics, Biology and Biochemistry,University of Torino

Primary Hyperoxaluria Type 1 (PH1) is a rare autosomal recessive disease, caused by germinal mutations on the AGXT gene encoding liver peroxisomal alanine-glyoxylate aminotransferase (AGT), a pyridoxal 5'-phosphate-dependent enzyme responsible for glyoxylate detoxification. The AGT deficiency leads to glyoxylate accumulation, thus leading to deposition of calcium oxalate crystals, first in the kidney and urinary tract, and then in the whole body [P. Chocat, et al, Nephrol Dial Transplant. 2012].
Here, we report the advancements of our research on the approaches proposed in the Telethon application: 1) study of the correlation between the AGXT genotype, the enzymatic phenotype and the clinical phenotype of PH1 patients, 2) planning of an enzyme enhancement therapy, 3) development of an enzyme administration therapy.
Results of the second year of the study:
1) the molecular defects of the W108R, S158L, G161S, G161C, D183N, S187, S218L, P319L, and G350D AGT variants have been characterized by means of biochemical (kinetic, spectroscopic, structural) and/or cell biology analyses; on the basis of the results, proposals for pharmacological agents able to counteract the adverse effect of these mutations have been advanced [E.Oppici, et al, Mol. Gen. and Metab. 2012]. In addition, the impact of G47R and S81L mutations either alone or in combination with G170R mutation has been elucidated and interpreted on the basis of clinical data of homozygous and compound heterozygous patients. In order to obtain updated clinical data, a revision of the entire cohort was made, and the clinical phenotype of mutated patients was updated to September, 2012.
2) the effect of aminoxyacetic acid (AOA), a known competitive inhibitor of AGT, on the expression of pathogenic AGT variants characterized by folding defects (G41R and G41V) has been analyzed in a cellular model system of the disease. AOA resulted to increase the specific activity of the variants in a dose-dependent manner, thus suggesting that it behaves as pharmacological chaperone. These results provide a promising step forward in the design of molecules acting as enzyme enhancement therapeutics for PH1.
3) A Tat-AGT fusion protein, made up of the AGT in fusion with a cell penetrating peptide (TAT peptide), has been constructed and purified. This molecule retains significant catalytic activity and is able to enter into mammalian cells and to productively detoxify peroxisomal glyoxylate [A. Roncador, et al, Int. J. Pept. Res. Ther. 2012]. These data, together with preliminary evidence for the formation of conjugates between AGT and polymeric nanoparticles, pave the way for the development of an enzyme administration therapy for PH1.

ABSTRACT N. 210

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SITIA ROBERTO**	
Telethon grant N.	**GGP11077**	
Total budget €	**343.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

PROTEOSTASIS IN THE EARLY SECRETORY COMPARTMENT AS A PATHOGENETIC MECHANISM AND THERAPEUTIC TARGET

Anelli Tiziana (1,2), Cortini Margherita (1,2), Fagioli Claudio (2), Mossuto Maria Francesca (1,2), Sannino Sara (2), van Anken Eelco (1,2), Fra Anna (3), Lougaris Vassilis (4), Plebani Alessandro (4), Sitia Roberto (1,2)

(1) University Vita Salute San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy, Tel. 02.26434722, Fax 02.26434723, E-mail: sitia.roberto@hsr.it
(2) Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan
(3) Department of General Pathology and Immunology, Brescia University
(4) Department of Pediatrics, Brescia University

Proteostasis defines the equilibrium between protein synthesis, folding, transport and degradation, necessary for cell survival and altered in many genetic diseases. Non-native secretory proteins are retained in the early secretory compartment and eventually degraded to limit cytotoxic aggregation and ER stress. ERp44 is a multifunctional chaperone of the PDI family, involved in quality control (retention of unpolymerized glycoproteins), redox homeostasis (regulation of Ero1 oxidases) and Ca2+ signaling (IP3R1 inhibition). In ERp44, a C-tail connects domains a and b' and regulates substrate binding/release. We showed that pH modulates its movements in vitro and in vivo. At pH 6.5 (as in cis-Golgi) the C-tail opens simultaneously exposing the substrate binding site and C-terminal RDEL motif. ERp44-client complexes bind to KDELR and are retrieved back to the ER: here neutral pH may favor dissociation of ternary complexes. We identified 3 residues in the C-tail/a domain interface and 2 conserved histidines at the b'/C-tail boundary as parts of the pH sensor machinery (Vavassori et al, submitted). Collaborative work with the Luini lab (TIGEM) suggests that ERp44 also mediates KDELR signaling, regulating ER-Golgi transport. Thus, the ER-Golgi pH gradient integrates quality control, redox homeostasis and KDELR-dependent signaling. Concerning Ca2+ signaling, our data show that ERp44 partly accumulates in mitochondrial associated ER membranes, binding IP3R1 (Anelli et al., 2012, ARS). To define the ERp44 interactome and movements, we extended it with a Halo-tag. This moiety binds covalently fluorescent or insoluble ligands, allowing 'in vivo chase' or co-immunoprecipitation experiments. As to the generation of inducible KO mice, a validated ES clone is being tested for germ line transmission.
Selective IgM Deficiency is a pediatric immunodeficiency of unknown etiology, characterized by low serum IgM but normal IgG. Being IgM a substrate of ERp44 and ERGIC53, a wider view of the ERp44 interactome could shed light on SIgMD pathogenesis. Seven patients (2 with undetectable, 5 with low IgM) were followed at the

Immunology Unit, Paediatric Clinic, University of Brescia. Most patients had a mild history of upper respiratory infections, except for one that suffered from an invasive pneumococcal infection at the age of 1. In the cases with low IgM, levels progressively decreased over the years. IgG and IgA levels, blood IgM+ B cell counts, lymphocyte subpopulations and responses to immunization were normal. No mutations were detected in the μ and J coding regions. We have obtained EBV-transformed B cell lines. Patient-derived lines secrete IgM at levels comparable to controls. Patients' B cells are being induced to differentiate in vitro, to determine whether the expression of the IgM secretory machinery is impaired in SIgMD.

ABSTRACT N. 211

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SVELTO MARIA**	
Telethon grant N.	**GGP12040**	
Total budget €	**114.400**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

STATINS AS POTENTIAL THERAPEUTIC AGENTS FOR HANDLING NEPHROGENIC DIABETES INSIPIDUS

Procino Giuseppe (1), Milano Serena (1), Carmosino Monica (1), Nicoletti Maria Celeste (1), Wess Jürgen (2), Svelto Maria (1)

(1) Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari, Via Orabona 4, 70126, Bari, Tel. 080.5443328, Fax 080.5443388, E-mail: maria.svelto@uniba.it
(2) National Institute of Diabetes and Digestive and Kidney Disease, NIH, Bethesda, MD, USA

X-linked nephrogenic diabetes insipidus (X-NDI) is a rare disease characterized by resistance of the kidney to the action of the antidiuretic hormone arginine-vasopressin (AVP) and is accompanied by severe polyuria and risk of dehydration. It is caused by inactivating mutations in the AVP type 2 receptor (V2R) gene that prevent the AVP-induced trafficking of the water channel AQP2 to the luminal plasma membrane of the kidney collecting duct (CD), and, consequently, reduces kidney concentration ability. In addition, as AVP also regulates AQP2 gene transcription, AQP2 protein is very low in NDI. This represents one of the principal limitations toward the establishment of an AQP2-targeted therapy that could bypass AVP signaling in NDI patients. Even if expressed at the plasma membrane, the residual amount of AQP2 expressed by an NDI patient might be insufficient to guarantee proper water reabsorption. These considerations prompted us to explore alternative signaling pathways that could restore physiological levels of AQP2 in the CD.
One of the best candidates that could fulfill this purpose is the secretin receptor (SCTR), a G-proteins-coupled receptor (GPCR) that, like V2R, elevates intracellular cAMP levels upon binding its ligand secretin (SCT), a hormone physiologically released by the intestinal mucosa.
We report here that SCTR is functionally expressed at the basolateral membrane of kidney collecting duct principal cells.
To investigate a possible beneficial effect of SCT on NDI phenotype, X-NDI mice, the only viable mouse model of X-linked NDI, were subcutaneously infused with SCT (2.5 mmol/Kg/day, via osmotic minipumps) for 14 days. Urinary parameters were not altered in SCT-infused animals. Interestingly, however, SCT significantly increased AQP2 levels in the CD, although the protein was mostly accumulated in the intracellular storage vesicles and hardly detectable at the plasma membrane.
We previously reported that fluvastatin (Flu) treatment, mimicking the action of AVP, transiently increases AQP2 abundance at the luminal membrane of the kidney CD in vivo in wild type mice in the absence of AVP stimulation.
SCT-infused X-NDI mice were injected intraperitoneally with Flu (50 mg/Kg). Diuresis and urine osmolality were monitored for 6 hours after Flu injection. Interestingly, Flu was able to potentiate the effect of SCT: cumulative diuresis was reduced by nearly 90% in mice treated with SCT+Flu and, accordingly, urine osmolality almost doubled in the same interval of time. Immunofluorescence confirmed that SCT increased intracellular stores of AQP2 and the addition of Flu promoted AQP2 accumulation at the plasma membrane.
Taken together these data strongly indicate that the combination of SCT and Flu might represent a new and effective pharmacological treatment for X-NDI.

ABSTRACT N. 212

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MATARESE GIUSEPPE**	
Telethon grant N.	**GJT08004**	
Total budget €	**100.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

LEPTIN, METABOLIC STATE AND NATURAL REGULATORY T CELLS: CELLULAR AND MOLECULAR BASIS FOR A NOVEL IMMUNE INTERVENTION IN TYPE 1 DIABETES

Matarese Giuseppe, De Rosa Veronica, Procaccini Claudio, Galgani Mario

Istituto di Endocrinologia e Oncologia Sperimentale (IEOS) del CNR, Via Pansini 5 80131 Napoli, Tel 081.7464580, E-mail: giuseppe.matarese@cnr.it

Type 1 diabetes is characterized by autoimmune destruction of pancreatic ß-cells in genetically susceptible individuals. Triggers of islet autoimmunity, time course and the precise mechanisms responsible for the progressive ß-cell failure are not completely understood. The recent escalation of obesity in affluent countries has been suggested to contribute to the increased incidence of type 1 diabetes. Understanding the link between, metabolism and immune tolerance could lead to the identification of new markers for the monitoring of disease onset and progression. We studied several immune cell subsets and factors with high metabolic impact as markers associated with disease progression in high-risk subjects, type 1 diabetes patients at onset, 12 and 24 months after diagnosis. A multiple correlation matrix among different parameters was evaluated statistically and assessed visually on two-dimensional graphs. Markers to predict residual ß-cell function up to one year after diagnosis were identified in multivariate logistic regression models.
The meta-immunology profile changed significantly over time in patients and a specific signature associated with worsening disease was identified. A multivariate logistic regression model measuring age, body mass index (BMI), fasting C-peptide, number of circulating CD3+CD16+CD56+ cells and the percentage of CD1c+CD19-CD14-CD303- type 1 myeloid dendritic cells (mDC1s) at disease onset had a significant predictive value. The identification of a specific meta-immunology profile that predicts residual ß-cell function through monitoring of the meta-immunology status of disease can help clinical decision and therapeutic guidance in type 1 diabetes, possibly leading to a better prognosis.

ABSTRACT N. 213

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MASIELLO PELLEGRINO**	
Telethon grant N.	**GJT08016**	
Total budget €	**50.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

PROTECTIVE EFFECTS OF PLANT COMPONENTS AGAINST CYTOKINE-INDUCED PANCREATIC BETA-CELL DYSFUNCTION AND DEATH AND EXPLORATION OF THE UNDERLYING MECHANISMS

Novelli Michela (1), Beffy Pascale (2), Menegazzi Marta (3), Porozov Svetlana (1), Martino Luisa (1), Masini Matilde (1), De Tata Vincenzo (1), Migliorini Paola (4), Marchetti Piero (4), Masiello Pellegrino (1)

(1) Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, Università di Pisa, Via Roma 55 , 56126 Pisa, Tel. +39.050.2218571, Fax +39.050.2218557, E-mail: pellegrino.masiello@med.unipi.it
(2) Istituto di Fisiologia Clinica, CNR, Pisa
(3) Dip. di Scienze della Vita e della Riproduzione, Università di Verona
(4) Dip. di Medicina Clinica e Sperimentale, Università di Pisa

Overall objective: To develop a new therapeutic approach for protection of pancreatic beta cells in type 1 diabetes, based on non-peptidyl vegetal compounds.
Background/aims: We previously showed that the extract of Hypericum perforatum (St. John's wort, SJW) and its component hyperforin (HPF), are potent inhibitors of cytokine-induced STAT-1 and NF-kB activation and prevent beta-cell death in INS-1E cell line, as well as in isolated rat and human islets. As STAT-1 pathway is involved in Th1 differentiation of T-cells, these compounds might also exert immunomodulatory effects.
Aims of the present report are: A) to further clarify the mechanisms

of the regulatory activity of SJW and HPF on cytokine signaling pathways in INS-1E cells; B) to assess their ability to counteract cytokine-driven changes in the expression of STAT-1 and NF-kB target genes involved in inflammatory response and apoptosis; C) to screen microarray profiling of microRNAs in beta cells exposed to cytokines and/or vegetal compounds; D) to test the immunomodulatory role of SJW and HPF on peripheral blood mononuclear cells (PBMC).
Methods: In INS-1E cells, exposed to cytokine mixtures for various time periods with/without SJW extract (1-2 microg/ml) or HPF (1-2 micromol/l), the phosphorylation state of various components of STAT-1, NF-kB and MAPK pathways was evaluated by western blotting. RNA was extracted with the mirVana miRNA isolation kit and used for RT-qPCR gene expression analysis (large RNA) and miRNA profiling.
Results: Aim A. Cytokine-induced STAT-1 phosphorylation in both tyrosine and serine residues was significantly reduced by SJW or HPF in a dose-dependent manner. These compounds prevented NF-kB activation by hindering phosphorylations of the p65 subunit and the inhibitory subunit IkB activating kinase and also modulated MAPK pathway through dose-dependent restriction of ERK1/2, p38 MAPK and JNK phosphorylations. Aim B. SJW and HPF abolished cytokine-induced mRNA expression of pro-inflammatory genes, such as iNOS, CXCL9, CXCL10, ICAM-1, COX-2. The vegetal compounds were also able to partially correct the cytokine-induced unbalance between anti- and pro-apoptotic factors, mainly by preventing up-regulation of pro-apoptotic proteins (e.g., DP5, PUMA, BAK). Aim C. Several miRNA were differentially expressed in cells exposed to cytokines as compared to control cells. Some of these changes were reversed in the presence of the vegetal compounds (in particular SJW), but in most cases the function of these miRNA in beta cells is unknown. Aim D. In stimulated PBMC, expression of T-bet and CXCR3-A, markers of Th1 differentiation, was down-regulated by SJW and HPF after 4-h incubation, whereas that of GATA-3 and CXCR3-B, markers of Th2 polarization, was up-regulated or unchanged.
Conclusions: SJW extract and HPF are confirmed to represent promising pharmacological tools for prevention of beta cell loss in type 1 diabetes and to exert immunomodulatory effects in vitro.

ABSTRACT N. 214

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FALCONE MARIKA MARIA CATERINA**	
Telethon grant N.	**GJT08017**	
Total budget €	**124.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

EXPLOITING THE THERAPEUTIC POTENTIAL OF INVARIANT NKT CELLS IN TYPE 1 DIABETES

Di Pietro Caterina (1), Nichols Kim (2), Caielli Simone (1), Falcone Marika (1)

(1) Experimental Diabetes Unit, San Raffaele Scientific Institute, L30 LottoQ, Via Olgettina 60, 20132, Milan, Italy, Tel. 02.2643.2317/2914, E-mail: falcone.marika@hsr.it
(2) Pediatric Oncology, Children s Hospital of Philadelphia, Philadelphia, USA

Background: Invariant NKT cells (iNKT) represent a unique subset of innate lymphocytes that play a dual role and exert either pro-inflammatory or regulatory function crucial to maintain T cell tolerance and prevent autoimmune diseases like T1D. Both iNKT cell functions are mediated through crosstalk between iNKT cells and myeloid dendritic cells (mDCs). Our research group demonstrated that genetic susceptibility to autoimmune diabetes in the NOD mice is linked to a genetic defect of the Slamf1 gene that impairs SLAM expression on myeloid DCs (mDCs) and negatively affects peripheral differentiation of regulatory NKT2 cells.
Objective: Our goal is to restore mechanisms that drive differentiation of regulatory NKT cells in diabetic mice and to verify whether the same genetic NKT cell differentiation defect is present in patients affected by T1D. The final goal is to design new therapeutic approaches for T1D based on restoration of regulatory NKT cell function.
Specific aim 1. To restore NKT2 differentiation in autoimmune-prone NOD mice by providing the necessary SLAM-induced co-stimulatory signals on myeloid dendritic cells (mDC). We successfully clone the murine SLAM gene into the MIGR1 retroviral vector that contains an internal ribosome entry site (IRES) linked to GFP transgene. We demonstrated that trasduction with the SLAM-MIGR1 vector completely restored the expression level of SLAM molecules on mDC of NOD mice. Our preliminary experiments indicate that SLAM-transduced DCs of NOD mice were still unable to induce regulatory NKT2 differentiation. We are currently testing whether this defect is related to defective expression of SLAM molecules on NOD NKT cells (that is required to obtain the homotipic SLAM-SLAM interaction) or to in vitro culture conditions that bias the cytokine phenotype and function of NKT cells towards a predominant effector NKT17/NKT1 phenotype.
Specific aim 2. To assess the capacity of regulatory NKT2 cells and NKT2-inducing mDC to prevent and/or treat T1D in pre-clinical models. Here we will assess the capacity of SLAM-MIGR1-transduced hematopoietic cells of NOD mice to promote differentiation of regulatory NKT2 cells in vivo and to prevent autoimmune diabetes. We have started experiments of chimera generation to replace irradiated of NOD mice with hematopoietic cells in vitro transduced with the SLAM-MIGR1 vector.
Specific aim 3. To analyze whether lack of co-stimulatory signals on mDC is responsible for defective NKT2 differentiation in T1D patients. We measured SLAM expression on mDC derived from blood monocytes of T1D patients (n=6) and controls (n=6). Although we observed a reduced expression of SLAM on DCs of T1D patients, our results were not statistically significant. In the last year of the research project we will analyse a larger cohort of T1D and patients (n=20) to verify whether mDCs carry a genetic defect of SLAM expression that could affect regulatory NKT2 differentiation.

ABSTRACT N. 215

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FEDERICI MASSIMO**	
Telethon grant N.	**GGP08065**	
Total budget €	**263.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

TESTING TIMP3 AS A SWITCH TO BLOCK METABOLIC AND VASCULAR COMPLICATIONS OF OBESITY

Federici Massimo, Casagrande Viviana, Cavalera Michele, Mavilio Maria, Stohr Robert, Menghini Rossella

Dipartimento di Medicina dei Sistemi, Università degli Studi di Roma Tor Vergata, Via Montpellier 1, 00133 Roma. Italia

TIMP3 regulates Fatty Acid Metabolism, Atherosclerosis progression and Survival in ApoE knockout mice. TIMP3 is reduced in the muscle and carotid plaques from patients with Type 2 Diabetes but if TIMP3 alters glucose and lipid metabolism is unclear. We analyzed ApoE-/-(EKO) and ApoE-/-Timp3-/- (ET3KO) mice by monitoring the survival rate; lipids, metabolites and atherosclerotic plaques with LC-MS metabolomics and conventional staining; physiological values by calorimetric cages. ET3KO mice revealed a significant higher rate of sudden death compared to EKO mice during a follow up of 12 months (N=18 per group; p<0.001); Sudan-IV and Oil-Red staining of ET3KO aorta and aortic root displayed increased positive staining (40% increase, p<0.01) compared to EKO. Moreover, ET3KO mice showed higher levels of total cholesterol, HDL-cholesterol and triglycerides (p<0.05 for all). Furthermore, ET3KO mice exhibited lower heart rate (p<0.01), higher systolic and diastolic pressures (p=0.06; p<0.05), higher O2 consumption and CO2 production, and lower respiratory exchange ratio compared to EKO mice (p<0.0001 for all). These data suggested a different use of metabolic substrates; specifically we found increased levels of C14:OH, C16:OH and C18:OH (p<0.01 for all) in the blood of ET3KO mice, together with higher amount of arginine (p<0.05) and histidine (p<0.01). No differences in food and water intake were found. Western blot analysis showed reduced pThr172 AMPK phosphorylation and increased pSer473 AKT phosphorylation in skeletal muscle from ET3KO compared to EKO mice (p<0.05 for all), suggesting that lipid oxidation may be dysfunctional in ET3KO mice. Loss of timp3 accelerates atherosclerosis and causes higher amount of atherosclerotic plaques, increased sudden death, plus defects in intermediary metabolism. The latter results in accumulation in the circulation of hydroxylated long-chain fatty acids that may complicate atherosclerotic plaque composition or perturb heart metabolism.

ABSTRACT N. 216

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BEGUINOT FRANCESCO**	
Telethon grant N.	**GGP09012**	
Total budget €	**245.400**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

PREP1 GENE FUNCTION IN TYPE 2 DIABETES

Oriente Francesco, Cabaro Serena, Griffo Elisabetta, Auriemma Fabiana, Beguinot Francesco

Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli, Via S. Pansini 5, 80131, Napoli, Tel. 081.7463248, Fax 081.7463235, E-mail: beguino@unina.it

Prep1 is an homeodomain transcription factor belonging to the TALE proteins, also including Pbx and Meis, playing an essential role in hematopoiesis, organogenesis and development. Prep and Meis proteins contain homologous conserved Pbx-Interacting-Motifs (HR1 and HR2 domains) and form transcriptionally active complexes with Pbx. DNA bound Meis/Prep-Pbx complexes, in turn, bind and modify the activity of other Hox and non-Hox homeodomain transcription factors. Previous studies have indicated that Prep1 hypomorphic mice have a complex metabolic phenotype. One key feature is increased insulin sensitivity in skeletal muscle accompanied by protection from streptozotocin-induced diabetes. Both in vivo and in vitro studies directed to clarify the molecular basis of these effects have shown that in muscle decreased Prep1 levels are followed by a reduction of p160. The absence of p160 increases the PGC1a-mediated Glut4 expression stimulating glucose uptake in skeletal muscle cells. More recent data have shown that Prep1i/+ mice feature increased glycogen content and reduced gluconeogenesis in liver, which does not involve a p160/PGC-1-dependent mechanism, but is due to an increase of insulin-stimulated insulin receptor and IRS1/2 tyrosine phosphorylation. Interestingly, Prep1i/+ mice show also decreased hepatic triglyceride levels. Here, we have investigated the function of the Prep1 gene on hepatic lipogenesis.
Serum triglycerides and hepatic expression of the lipogenetic enzyme Fatty Acid Synthase (FAS) were found significantly decreased in Prep1i/+ mice. Western Blot analysis have shown increased phosphorylation of PKCzeta, LKB1, AMPK and ACC, controlling FAS expression and triglycerides production in liver. Protein and mRNA levels of the lipid phosphatase SHIP2, an inhibitor of PI3Kinase/PKCzeta signaling, were reduced by 40% in the liver of Prep1i/+ mice. Consistent with these data, HepG2 cells overexpressing Prep1 display increased triglyceride levels and FAS expression, while PKCzeta, LKB1, AMPK and ACC phosphorylation is strongly reduced. This effect is due by the upregulation of SHIP2 mediated by the Prep1/Pbx1 complex. To assess the consequences of the Prep1-mediated lipogenesis, we induced hepatic steatosis by feeding WT and Prep1i/+ mice a methionine and choline-deficient (MCD) diet. In Prep1i/+ mice, MCD diet induced a minor increase of aminotransferase and triglyceride content compared to the WT mice. Also the histological changes as steatosis, inflammation, and hepatocyte necrosis are less evident to those observed in WT mice, indicating that Prep1 reduction mitigate the effect of the MCD diet-induced hepatic steatosis.
These data suggest that Prep1-deficiency reduces lipotoxicity by increasing PKCzeta/AMPK signaling and ameliorates steatosis giving a rationale to investigate Prep1 as possible new therapeutic agents in preventing fatty liver progression to nonalcoholic steatohepatitis (NASH).

ABSTRACT N. 217

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BAUCE BARBARA**	
Telethon grant N.	**GGP09293**	
Total budget €	**246.900**	
Centres: **3**	*Duration (yrs):* **2**	*Starting year:* **2009**

NOVEL GENES AND CLINICAL-HISTOPATHOLOGIC FINDINGS IN FAMILIES AFFECTED BY ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY

Basso Cristina (2), Rampazzo Alessandra (3), Rigato Ilaria (1), Perazzolo Marra Martina (1), Carturan Elisa (2), Pilichou Kalliopi (2), Smaniotto Jessica (3), Rizzo Stefania (2), De Bortoli Marzia (3), Calore Martina (3), Thiene Gaetano (2), Bauce Barbara (1)

Clinica Cardiologica, Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari, Università di Padova, Via Giustiniani 2, 35100 Padova, Tel. 049.8218642, Fax 049.0211802, E-mail: barbara.bauce@unipd.it
(2) Patologia Cardiovascolare, Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari, Università di Padova, Padova
(3) Dipartimento di Biologia, Università di Padova, Padova

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a cardiac disease characterized by electrical instability that can lead to sudden death. As mutations causing ARVC have been mainly identified in genes encoding for desmosomal proteins, it might be considered as a disease of the desmosome.
Results. Clinical and genetic screening of ARVC probands and family members. A total of 110 unrelated index cases and 219 family members were screened for mutations of desmosomal genes Plakofillin-2 (PKP2), Desmoplakin (DSP) and Desmoglein-2 (DSG2) and 137 were found to be mutation carriers. The degree of penetrance was lower in the groups with a single gene mutation than in the multiple mutations group. Finally, analysis of a pediatric population with desmosomal gene mutations showed that clinical signs usually develop during adolescence and young adulthood and that contrast-enhanced cardiac magnetic resonance provides relevant diagnostic information.
Identification of a novel ARVC gene. We speculated that alfa T-catenin, encoded by CTNNA3, might carry mutations in ARVC patients. Alpha T-catenin binds PKPs and this binding contributes to the formation of the area composita, which strengthens cell-cell adhesion in cardiomyocytes. We screened for mutations CTNNA3 gene in 76 ARVC patients negative for mutations in the ARVC desmosomal genes. Mutations c.281T>A (p.V94D) and c.2293_2295delTTG (p.del765L) were identified in two probands. Yeast two-hybrid and cell transfection studies showed that the interaction between the p.V94D mutant protein and beta-catenin was affected, whereas the p.del765L mutant protein showed a much stronger dimerisation potential and formed aggresomes in HEK293T cells. These findings might point to a causal relationship between CTNNA3 mutations and ARVC. This first report on the involvement of an area composita gene in ARVC shows that the pathogenesis of this disease extends beyond desmosomes.
Intercellular junctions ultrastructural and immunohistochemical abnormalities in myocardial specimens. To better understand the pathogenesis of ARVC, we used immunohistochemistry to characterize the distribution of junctional proteins at intercalated disks. We evaluated if mutant desmosomal proteins are expressed at intercalated disks and if a mutation in a single desmosomal protein could affect the distribution of other proteins within cell-cell junctions. We have shown diminished immunoreactive signal for PG at intercalated disks in patients with DSP, PKP2, DSP2 and PG mutations. PG signal was reduced not only in RV regions with pathological changes, but also in the LV free wall and IV septum histologically unremarkable. Reduced PG signal seemed to be specific for ARVC, as compared with cases of end-stage heart failure due to other cardiac diseases. Further studies are required to validate specificity and sensitivity of the test, but these observations suggest that reduced signal for PG in a heart biopsy may have a role in the diagnosis.

ABSTRACT N. 218

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**PIERONI MAURIZIO**	
Telethon grant N.	**GGP10186**	
Total budget €	**336.000**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2010**

IDENTIFICATION OF GENETIC, ELECTROANATOMICAL AND STRUCTURAL PREDICTORS OF MALIGNANT VENTRICULAR ARRHYTHMIAS IN PATIENTS WITH BRUGADA SYNDROME

Pieroni Maurizio (1), Antonio Oliva (2), Gemma Pelargonio (3), Camporeale Antonia (3), Partemi Sara (2), Notarstefano Pasquale (1), Pagans Sara (4), Pascali Vincenzo (2), Bolognese Leonardo (1), Brugada Ramon (4)

(1) Cardiovascular Department ASL8 Arezzo San Donato Hospital Via Pietro Nenni, 20 52100 Arezzo, Italy
(2) Institute of Forensic Medicine, Catholic University Rome, Italy
(3) Cardiovascular Department, Catholic University Rome, Italy
(4) Cardiovascular Genetic Department, Girona, Spain

Background
In the first clinical part of our project we sought to evaluate the presence and the substrate of three dimensional-electroanatomic map (3D-EAM) abnormalities in patients with Brugada syndrome (BrS). We evaluated 3D-EAM and 3D-EAM-guided right ventricular endomyocardial biopsies in patients with BrS.
Methods
We have enrolled in this part of the study 13 consecutive patients (11M, 49±8 year-old) with a diagnosis of BrS according to current criteria. In particular, all patients had a type I ECG pattern at rest (n=9) or after flecainide challenge (n=4). All patients were submitted to 3D-EAM and 3D-EAM-guided endomyocardial biopsy. In pa-

tients with normal 3D-EAM, biopsies were drawn from both septal-apical region and right ventricular outflow tract (RVOT).
Results
Clinical presentation included sustained polymorphic ventricular tachycardia in 7 patients, syncope in 3, while 3 patients were asymptomatic. Eleven patients (84%) showed an abnormal right ventricular 3D-EAM. Eight patients presented low-voltage areas in the RVOT, in 4 cases associated with free wall involvement, and in 2 cases with postero-basal and inferior wall involvement. The remaining 3 patients presented low-voltage areas in the free wall (n=2) or in the posterobasal segment. Among patients with abnormal 3D-EAM, histology showed pathologic findings in 9 patients (81%). In particular we documented fibrofatty replacement in two patients with RVOT low-voltage areas, myocarditis in 5 patients with low-voltages in the free wall (in 3 cases with RVOT involvement) and non-specific cardiomyopathic changes, in two patients with septal RVOT and posterobasal segment low-voltage areas. Normal myocardial tissue was observed in biopsies from 2 patients with normal 3D-EAM, and in 2 patients with RVOT abnormal voltage areas. No significant correlation was observed between clinical and ECG features and neither 3D-EAM nor histologic findings. Programmed electrical stimulation induced ventricular fibrillation in two patients, one with RVOT involvement.
Conclusions
We found a high prevalence of abnormal 3D-EAM among BrS patients, with RVOT being the most frequently involved segment. Abnormalities of 3D-EAM reflected an underlying myocardial disorder in 81% of patients, thus reinforcing the notion that BrS is not a pure electrical disorder. The identification of abnormal voltage areas and the corresponding myocardial substrate may influence both prognosis and treatment, including ablation strategies.

ABSTRACT N. 219

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TARONE GUIDO**	
Telethon grant N.	**GGP12047**	
Total budget €	**182.600**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

MELUSIN GENE THERAPY: A POSSIBLE APPROACH TO PREVENT CARDIOMYOPATHY

Tarone Guido, Brancaccio Mara, Vitale Nicoletta, Rubinetto Cristina, Cimino James

Università di Torino, Dipartimento di Biotecnologie Molecolari e Scienze per la Salute, Via Nizza 52, 10126 Torino, Tel. 011.6706433, Fax 011.6706432, E-mail: guido.tarone@unito.it

Cardiomyopathies are life threatening diseases caused in large part by hereditary genetic mutations leading to impaired heart function. Since these pathologies are generated by several possible mutations affecting different genes, conventional gene therapy targeting each specific mutation is not always feasible. Mutation-independent gene therapy approaches could be greatly advantageous being, in principle, applicable to cardiomyopathies generated by different, as well as not yet identified mutations. Here we plan to test the efficacy of a gene therapy approach based on overexpression of melusin gene coding for a muscle-specific protein with chaperone activity. Chaperones are naturally occurring proteins that recognize a broad panel of proteins partially damaged by stress conditions or genetic mutations allowing them to retain correct function. We have previously demonstrated that melusin is selectively expressed in muscles and heart and it is required for the heart response to stress stimuli such as aortic stenosis. Moreover, melusin overexpression effectively counteracts cardiac dilation and heart failure induced by aortic stenosis and myocardial infarct. Here we propose to use melusin gene therapy as mutation-independent approach to buffer the cellular stress induced by genetic mutations in mouse models of familial cardiomyopathy.

ABSTRACT N. 220

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**PRIORI SILVIA GIULIANA**	
Telethon grant N.	**GGP11141**	
Total budget €	**465.600**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

MUTATIONS OF CARDIAC CALSEQUESTRIN AND CARDIAC ARRHYTHMIAS: NOVEL INSIGHTS ON PATHOGENESIS AND THERAPY

Denegri Marco (1), Lodola Francesco (1), Boncompagni Simona (2), Liu Nian (3), Valle Giorgia (4), Volpe Pompeo (4), Protasi Feliciano (2), Napolitano Carlo (1,3), Priori Silvia G (1,3,5)

(1) Molecular cardiology, IRCCS Fondazione Salvatore Maugeri, Via Maugeri 10, 27100, Pavia, Italy, Tel. 0382.592049, Fax 0382.592059, E-mail: silvia.priori@fsm.it
(2) CeSI, Center for Research on Ageing & DNA, Department of Neuroscience and Imaging, G. d'Annunzio University, Chieti, Italy
(3) Cardiovascular Genetic Program, The Leon H. Charney Division of Cardiology, New York University School of Medicine, New York, USA
(4) Department of Biomedical Sciences, University of Padova, Padova, Italy
(5) Department of Molecular Medicine, University of Pavia, Pavia, Italy

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) is an inherited arrhythmogenic disease causing sudden, stress-induced, ventricular arrhythmias and syncopal spells. Sudden death can be the first manifestation and the mortality is up to 30% within the fourth decade of life. In previous Telethon-funded projects we discovered the gene for the autosomal dominant CPVT (RyR2) and we contributed to define its genetic epidemiology and pathophysiology through the establishment of registries and transgenic models. The project GGP11141 is aimed at achieving a major advance towards the development of a gene-based therapy for CPVT. We specifically focus on the autosomal recessive variant (CASQ2 mutations).
We first characterized our CASQ2 knock out (KO) model showing >90% loss of CASQ2 monomers, possibly because of enhanced degradation of the mutant protein. Electron microscopy in KO mice showed widening of the jSR lumen from 26±0.3 nm to 37±1.2 nm; p<0.001). The decrease of CASQ2 was associated with harmful arrhythmias (closely mimic those observed in patients) that are caused by triggered activity (TA) at the cellular level. In collaboration with dr. Auricchio (TIGEM) we engineered an AAV9 vector with WT CASQ2 and infected KO mice. After 35 weeks we demonstrated complete inhibition of arrhythmias and reversal of ultrastructural abnormalities (Denegri M et al Circ Res 2012). Since these results proved that the restoration of CASQ2 expression has a therapeutic potential, we investigated the effectiveness of AAV9 infection in the CASQ2-R33Q/R33Q mouse we previously developed, which harbors a mutation found in a CPVT family. First, we thoroughly characterized Ca2+ handling dynamics and demonstrated that R33Q/R33Q myocytes develop spontaneous Ca2+ releases leading to early (EAD) and delayed (DAD) afterdepolarizations. Cells also displayed a disruption of the CRUs architecture associated with fragmentation of Ca2+ waves. Together these findings delineate a novel arrhythmogenic mechanism in CPVT (Liu N et al. submitted). We then tested the effectiveness of our gene targeting strategy. CASQ2-R33Q/R33Q mice were studied at 26 and 52 weeks after infection. The incidence of ventricular tachycardia dropped from 87% (n=8) in controls to 17% (n=12) infected mice (p<0.005). Accordingly, DADs and TA were almost abolished (26 weeks: DADs: 0%, TA 0%, n=18; 52 weeks: DADs: 8%, TA: 8%, n=12). Finally, western blot and immunofluorescence assays indicated re-expression and correct CASQ2 localization.
CONCLUSIONS. Here we clearly show that AAV9-mediated re-expression of normal CASQ2 is an effective and long-lasting strategy to revert the functional abnormalities induced by CASQ2 mutations even when, as in the case of R33Q, the endogenous mutant is still present. Since AAV vectors have already been used in humans, our data can prelude the design of a clinical trial to test gene therapy of recessive CPVT.

ABSTRACT N. 221

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MONGILLO MARCO**	
Telethon grant N.	**GGP11224**	
Total budget €	**215.600**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

NOVEL OPTOGENETIC APPROACH TO INVESTIGATE ARRHYTHMOGENESIS IN DOMINANT CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA (CPVT)

Da Broi Francesca, Plazzo Anna Pia, Zaglia Tania, Mongillo Marco

Venetian Institute of Molecular Medicine, Via Orus, 2, 35129 Padova, Tel. 049.7923229 and Dept of Biomedical Sciences, University of Padova, Padova, Italy

The heart is densely innervated by sympathetic neurons (SN) that

regulate cardiac function during exercise and stress through beta adrenergic receptors (beta-ARs) activation. Increased cardiac sympathetic activity can lead to arrhythmias in CPVT, possibly via development of Ca2+ overload-dependent DADs under norepinephrine (NE) stimulation. The DAD would serve as arrhythmogenic focus, leading to the onset of triggered activity in groups of cardiac cells. Unbalanced sympathetic discharge to different regions of the heart has been associated with arrhythmia triggering (Arora, Am J Physiol Regul Integr Comp Physiol 2003), and alterations in presynaptic NE reuptake lead to catecholamine spillover in the failing myocardium (Esler, Hypertension 1988). These data support a model in which autonomic control of contractility occurs through direct interaction between the neurons and their target cardiomyocytes (CM).
The aim of this study is to investigate whether specific cell-cell interactions have a role in the dynamics of intercellular signaling between SN and CM. This will allow to understand how unbalanced SN activity can modify CM physiology and eventually lead to arrhythmic condition.
We set up co-cultures between neonatal rat CM and SN isolated from the superior cervical ganglia as in vitro model. Upon NGF treatment, SN extend their axons and establish direct contact with CM; NE-synthesizing terminals developed on SN at the contact site, where beta1-AR are enriched on the CM membrane.
We assessed intracellular beta-AR-dependent signaling by performing real-time cAMP and PKA activity imaging with the FRET-based biosensor EPAC1-camps or AKAR3, respectively. Stimulation of SN was achieved using KCl, bradykinin or by controlling SN membrane potential with a patch clamp pipette. Activation of a specific SN lead to cAMP increase in the interacting CM (EPAC1-camps ?R/R0= 0.056 ± 0.007, n=9, AKAR3 ?R/R0=0.048 ± 0.013, n=8), while no changes were detectable in neighbouring but not directly interacting CM.
To estimate the [NE] acting on the CM beta-AR at the contact site, we compared the amplitude of the FRET signal evoked by SN activation to that elicited by different [NE] administered to the cell bathing solution. These experiments were also performed in the presence of different doses of the beta-blocker propranolol allowing to estimate [NE] in the intermembrane cleft to be in the order of a hundred nM. This will allow to calculate the fraction of beta-ARs activated by the SN-released NE.
In conclusion, we provide evidence of the existence of a 'synaptic' contact between SN and CM that forms a high agonist concentration, diffusion-restricted space allowing potent activation of a small fraction of beta-AR on the CM membrane upon neuronal stimulation. This close interaction has the potential of fast control of local CM signalling, suggesting that SN control of restricted group of cells could generate arrhythmic focus in pathological condition.

ABSTRACT N. 222

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**CROTTI LIA**	
Telethon grant N.	**GGP09247**	
Total budget €	**383.700**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2009**

FROM MOUSE TO MAN, USING PHYSIOLOGY TO DEVELOP A GENE-SPECIFIC MANAGEMENT OF LETHAL LQT3 VARIANT OF LONG QT SYNDROME

Crotti Lia (1,2), Zentilin Lorena (3), Macedo Antero (3), Giacca Mauro (3), Schwartz Peter (1,2), Insolia Roberto (2), Girardengo Giulia (2), Besana Alessandra (5), Calvillo Laura (5), Montano Nicola (6), Wu Maddalena (4), Bari Vlasta (4), Porta Alberto (4)

Dipartimento di Cardiologia, IRCCS Fondazione Policlinico San Matteo, Piazzale Golgi 19, 27100, Pavia, Tel. 0382.501323, Fax 0382.501322, E-mail: l.crotti@smatteo.pv.it
(2) Sezione di Cardiologia, Dipartimento di Medicina Molecolare, Università di Pavia, Pavia
(3) International Center for Genetic Engineering and Biotechnolgy (ICGEB), Padriciano, Trieste
(4) Dipartimento di Tecnologie per la Salute, Istituto Ortopedico Galeazzi, Università degli Studi di Milano, Milano
(5) Laboratorio di Genetica Cardiovascolare, IRCCS Istituto Auxologico Italiano, Milano
(6) Dipartimento di Scienze Cliniche, Medicina Interna II, Ospedale L. Sacco, Università di Milano, Milano

The specific aim of this project is the development of a therapeutic strategy for LQT3 based on the selective and permanent knockdown of the SCN5A mutant allele through AAV vector-mediated expression of siRNAs targeted to the mutated sequence.
The experimental strategy takes advantage of the presence of a characterizing 9-bp deletion in the mutant allele (DeltaKPQ). On this basis, 5 different targets for silencing were selected, matching 19 or 29 nt of genomic sequence and spanning the deletion site. Corresponding oligos were cloned into an AAV vector backbone under the transcriptional control of Pol III U6 promoter. This vector contains a second transcriptional cassette expressing the fluorescent ZsGreen reporter protein, and allows to easily monitoring transduction efficiency.
High titer AAV6 viral particles were generated at the AVU Facility of ICGEB and tested in vitro in culture of neonatal cardiomyocytes obtained from knock-in mice, heterozygous for the highly malignant SCN5A-DeltaKPQ mutation. In the presence of 90% transduction efficiency, allele-specific real-time PCR analysis demonstrated that shRNA5, mediates silencing of the SCN5A alleles with a selective, although not absolute, effect towards the mutant allele.
Parallel electrophysiological analysis revealed reproducible normalization of the electrical abnormalities characterizing LQT syndrome. An AAV9-sh5, was generated and injected into neonatal mice. Quantitative molecular analysis performed after one month from injection, confirmed the in vivo silencing of the messenger RNA of the mutant allele at levels closed to 50% with respect to untreated or AAV control injected mice, without evident adverse effects. Similarly treated mice will be assessed for telemetric parameters and arrhythmic events in response to carbacol treatment.
Searching for a siRNA with a more selective specificity toward the Delta KPQ allele, we tested in vitro, in our neonatal cardiomyocyte culture model, 10 new target sequences. One out of these, siRNA-4734, induced 60% reduction of the levels of mutant allele and almost no effect on wild type (WT) allele, as assessed by differential real time quantification.
We will further exploit the potential of this siRNA sequence by AAV-mediated gene transfer in vivo in DeltaKPQ mice.
The SCN5A-DeltaKPQ mice model is a precious translational tool. We have exploited the availability of these mice to characterize the cardiac phenotype in the attempt of identifying predictors of risk.
Heart rate (HR), body temperature and the locomotor activity signals were continuously recorded by a telemetric system for 3 consecutive days, in 6 WT and 6 transgenic LQT mice. Animals were kept in a temperature-controlled room at 20–24°C, with constant light/dark cycle (light on from 6 AM to 6 PM). Circadian rhythm variation analysis was performed through the averaging of each 5 min for the whole 24 hours. The 24-hours were divided in two 12-hours periods, LIGHT (the quiet/sleep cycle) and DARK (the activity/wake cycle).
In the LQT group HR was lower during LIGHT than during NIGHT (497±70 vs 535±51 bpm; $p<0.05$). WT group behaved similarly (545±23 vs 571±40 bpm; $p<0.05$). The HR variability was larger in the LQT group than in the WT both during DARK (5453±781 vs 4185±1427 bpm; $p<0.05$) and LIGHT (5266±388 vs 4189±966 bpm; $p<0.05$).
LQT and WT mice showed a preserved HR circadian behavior. In addition, in the LQT mice HR variations were unusually large, thus suggesting an abnormal cardiac regulation.

ABSTRACT N. 223

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SANTUCCI ANNALISA**	
Telethon grant N.	**GGP10058**	
Total budget €	**233.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

SET UP OF EXPERIMENTAL MODELS OF ALKAPTONURIA AND PRECLINICAL TESTING OF THERAPEUTIC AGENTS FOR THE TREATMENT OF OCHRONOTIC ARTHROPATHY

Millucci Lia (1), Spreafico Adriano (1), Tinti Laura (1), Braconi Daniela (1), Ghezzi Lorenzo (1), Paccagnini Eugenio (2), Bernardini Giulia (1), Amato Loredana (1), Laschi Marcella (1), Lupetti Pietro (2), Orlandini Maurizio (1), Santucci Annalisa (1)

(1) Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, via Fiorentina 1, 53100, Siena, Tel. 0577.234958, Fax 0577.234903, E-mail: annalisa.santucci@unisi.it
(2) Dipartimento di Scienze della vita, Università degli Studi di Siena, Siena

Alkaptonuria (AKU) is an ultra-rare disease developed from the lack of homogentisic acid oxidase activity, causing homogentisic acid (HGA) accumulation that produces a HGA-melanin ochronotic pigment, of unknown composition. There is no therapy for AKU. Our aim was to verify if AKU implied a secondary amyloidosis.

Materials and Methods
Congo Red, Thioflavin-T staining and TEM were performed to assess amyloid presence in AKU specimens (cartilage, synovia, periumbelical fat, salivary gland) and in HGA-treated human chondrocytes and cartilage. SAA and SAP deposition was examined using immunofluorescence and their levels were evaluated in the patients' plasma by ELISA. 2D electrophoresis was undertaken in AKU cells to evaluate the levels of proteins involved in amyloidogenesis.
Results and future perspectives
AKU osteoarticular tissues contained SAA-amyloid in 7/7 patients. Ochronotic pigment and amyloid co-localized in AKU osteoarticular tissues. SAA and SAP composition of the deposits assessed secondary type of amyloidosis. High levels of SAA and SAP were found in AKU patients' plasma. Systemic amyloidosis was assessed by Congo Red staining of patients' abdominal fat and salivary gland. AKU is the second pathology after Parkinson's disease where amyloid is associated with a form of melanin. Aberrant expression of proteins involved in amyloidogenesis has been found in AKU cells. Our findings on alkaptonuria as a novel type II AA amyloidosis open new important perspectives for its therapy, since methotrexate treatment proved to significantly reduce in vitro HGA-induced A-amyloid aggregates (Millucci L, et al. Alkaptonuria is a novel human secondary amyloidogenic disease. Biochim Biophys Acta 2012;1822(11):1682-91. doi: 10.1016/j.bbadis. 2012.07.011).

ABSTRACT N. 224

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BALDINI ANTONIO**	
Telethon grant N.	**GGP11029**	
Total budget €	**326.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

PHENOTYPIC VARIABILITY AND GENE HAPLOINSUFFICIENCY

Caprio Cinzia (1), Fulcoli Filomena Gabriella (1), Franzese Monica (2), Angelini Claudia (2), Baldini Antonio (1)

(1) Istituto di Genetica e Biofisica "Adriano Buzzati-Traverso", CNR, Via Pietro Castellino, 111-80131 Napoli, Italy
(2) Istituto per le Applicazioni del Calcolo, "M. Picone" , CNR, Via Pietro Castellino, Napoli, Italy

The haploinsufficiency of Tbx1 and its mutation is a cause of DiGeorge syndrome, a multiple congenital anomaly syndrome that includes also an important adult phenotype. The gene encodes a transcription factor of the T-box family. The scope of the project is to investigate mechanisms leading to Tbx1 gene haploinsufficiency and reduce its phenotypic impact in vivo using drugs. With this project, we have linked at least some molecular functions of Tbx1 to interactions with a Swi-Snf-like chromatin remodeling complex and with histone methyltransferase enzymes. More recently, we have carried out genome-wide ChIP-seq assays to map binding sites of Tbx1 and correlate them with gene features and chromatin profiling. The results indicated that Tbx1 binding sites are mainly located distal to transcription start sites (64,2%), and that they strongly correlate with H3K4me1 enrichment (77,4%). Using quantitative assays, we also found that Tbx1 enrichment correlates positively with H3K4me1 at specific loci. Thus, we hypothesized that reduced dosage of Tbx1 may lead to target gene disregulation through insufficient H3K4me1 enrichment. We reasoned that treatment with a demethylase inhibitor may compensate for reduced dosage of Tbx1 and thus ameliorate the haploinsufficiency phenotype. Therefore, we treated mutant mice with the epi-drug tranylcypromine (TCP), a drug approved for human use, which is a potent inhibitor of Lsd1-mediated demethylation. The typical embryonic phenotype of Tbx1 haploinsufficiency is hypo/aplasia of the fourth pharyngeal arch artery at E10.5. Results showed that injection of TCP leads to a significant (P value=0.01), albeit partial rescue of aortic arch defects in embryos. In parallel, we experimented another strategy to phenotypic rescue based on our previous findings that loss of Tbx1 leads to reduced cell proliferation and enhanced cell differentiation in several target tissues. We tested whether reduced dosage of p53 (either genetically, using a p53 mutant, or pharmacologically using Pifithrin) may modify the Tbx1 mutant phenotype in vivo, using the same biological assay in vivo. Results showed a near complete rescue of the haploinsufficient phenotype in E10.5 embryos using genetic ablation of p53, and a less dramatic but still significant rescue using the drug (P=4.6*10-5).
Overall, our results indicate that the Tbx1 haploinsufficiency, morphogenetic phenotype can be at least partially corrected or prevented using drugs. Further refinement of our knowledge of mechanisms of action of Tbx1 will provide additional drug targets.

ABSTRACT N. 225

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**ARBUSTINI ELOISA**	
Telethon grant N.	**GGP08238**	
Total budget €	**194.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

LOSARTAN VS. NEBIVOLOL VS. THE ASSOCIATION OF BOTH ON THE PROGRESSION OF AORTIC ROOT DILATION IN MARFAN SYNDROME (MFS) WITH FBN1 GENE MUTATIONS: THE ON-GOING CLINICAL TRIAL

Serio Alessandra (1), Grasso Maurizia (1), Favalli Valentina (1), Disabella Eliana (1), Giorgianni Carmela (1), Regazzi Mario (2), Broglia Monica (2), Klersy Catherine (3), Dore Roberto (4), Arbustini Eloisa (1)

(1) Centro Malattie Genentiche Cardiovascolari, Fondazione IRCCS Policlinico San Matteo, Pavia
(2) Unità di Farmacologia e Farmacocinetica, Fondazione IRCCS Policlinico San Matteo, Pavia
(3) Direzione Scientifica, Fondazione IRCCS Policlinico San Matteo, Pavia
(4) Dipartimento di Radiologia, Fondazione IRCCS Policlinico San Matteo, Pavia

Introduction. Marfan Syndrome (MFS) is a rare genetic disease caused by mutations of the Fibrillin 1 gene (3:10,000 individuals). Aortic root aneurysm (>80% of cases) may complicate with aortic dissection when the root diameter approximates 5 cm. Since the key mediator of aortic wall damage is the Transforming Growth Factor-beta 1 (TGFß1) and in experimental animals TGFß1 blockade prevents structural damage and dilation of the aorta, Angiotensin II Receptor Blockers (ARB) exerting an anti-TGFß1 effect, are candidate to treatment of MFS [Shin GT, Am J Kidney Dis 2000; Fukuda N, Am J Hypertens 2000]. In the past, beta-blockers (BB) have been commonly used but their effectiveness in preventing aortic dissection is still debated.
Methods. We designed an open-label phase III study that includes 291 (231 for statistical power 90%, and 60 calculating a drop out of 20%) patients with MFS and proven FBN1 gene mutations and aortic root dilatation. The patients were randomized to Nebivolol, Losartan or the combination of both. Each patient underwent baseline evaluation, including ECG, echocardiogram (standard echo and additional aortic evaluation according to Roman's method and z-score calculation [Roman MJ, Am J Cardiol 1989]), and blood sample analysis, QoL assessment through the SF-36 questionnaire. Subsequent visits are every 12 months. The primary endpoint is the comparative evaluation of effects of the medical therapy on the progression of aortic root dilatation. Secondary end-points include the pharmacokinetics of the two drugs, comparative evaluation of serum levels of total and active TGFß1, quantitative assessment of the expression of the mutated gene, pharmacogenetic bases of drug responsiveness. The QoL evaluation in the three groups is being assessed. Statistical evaluation includes an interim analysis at month 24 and conclusive analyses at month 48. The local ethic committee approved the trial. The study is registered in the WHO register ClinicalTrials.gov (no. NCT00683124) and it is recognized by the Italian Drug Agency (EudraCT number 2008-001462-81).
Results. The sample size (net drop out, n=262) was reached in September 2012. The enrolment was formally closed and communicated to the local ethical committee in September 10, 2012. The observed drop was significantly lower than calculated (8% vs. 20%) due to the high adherence and participation to the project of patients and families. More than 50% of the patients completed the follow-up at 24 months. Interim analysis is ongoing.
Conclusions. The follow-up is ongoing as planned. To date, none of the patients worsened or developed acute aortic dissection. Both drugs are well tolerated without evidence of major side effects. Scheduled time for follow-up has to be respected before knowing the results in the three arms of the trial.

ABSTRACT N. 226

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MOSCHETTA ANTONIO**	
Telethon grant N.	**GGP08259**	
Total budget €	**205.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2008**

TARGETING NUCLEAR BILE ACID RECEPTOR FXR IN PROGRESSIVE FAMILIAL INTRAHEPATIC CHOLESTASIS

Moschetta Antonio (1), Modica Salvatore (1), Petruzzelli Michele (1,2), Bellafante Elena (1), Murzilli Stefania (1), Salvatore Lorena (1), Celli Nicola (3), Di Tullio Giuseppe (1), Palasciano Giuseppe (2)

(1) Laboratory of Lipid Metabolism and Cancer, Department of Translational Pharmacology, Consorzio Mario Negri Sud, Santa Maria Imbaro (CH), Tel. 0872.570344; Fax 0872.570299; E-mail: moschetta@negrisud.it
(2) Clinica Medica "A. Murri," Department of Internal and Public Medicine, University of Bari, Bari, Italy
(3) Environmental Sciences Center, Consorzio Mario Negri Sud, Santa Maria Imbaro (CH), Italy

Cholestasis is a liver disorder characterized by impaired bile flow, reduction of bile acids (BAs) in the intestine, and retention of BAs in the liver. The farnesoid X receptor (FXR) is the transcriptional regulator of BA homeostasis. Activation of FXR by Bas reduces circulating BA levels in a feedback mechanism, repressing hepatic cholesterol 7alpha-hydroxylase (Cyp7a1), the rate-limiting enzyme for the conversion of cholesterol to BAs. This mechanism involves the hepatic nuclear receptor small heterodimer partner and the intestinal fibroblast growth factor (FGF) 19 and 15. We investigated the role of activation of intestine-specific FXR in reducing hepatic levels of BAs and protecting the liver from cholestasis in mice. We generated transgenic mice that express a constitutively active FXR in the intestine. Using FXR gain- and loss-of-function models, we studied the roles of intestinal FXR in mice with intrahepatic and extrahepatic cholestasis.
Selective activation of intestinal FXR induced FGF15 and repressed hepatic Cyp7a1, reducing the pool size of BAs and changing the BA pool composition. Activation of intestinal FXR protected mice from obstructive extrahepatic cholestasis after bile duct ligation or administration of alpha- naphthylisothiocyanate. In Mdr2-/- mice, transgenic expression of activated FXR in the intestine protected against liver damage, whereas absence of FXR promoted progression of liver disease. Activation of FXR transcription in the intestine protects the liver from cholestasis in mice by inducing FGF15 expression and reducing the hepatic pool of BA; this approach might be developed to reverse cholestasis in patients.

ABSTRACT N. 227

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FABRIS LUCA**	
Telethon grant N.	**GGP09189**	
Total budget €	**218.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

EPITHELIAL-MESENCHYMAL TRANSITION AND CROSS-TALK IN THE DEVELOPMENT OF LIVER FIBROSIS IN CONGENITAL FIBROCYSTIN DEFECTS (CONGENITAL HEPATIC FIBROSIS)

Fabris Luca (1), Locatelli Luigi (2), Cadamuro Massimiliano (1), Spirli Carlo (3), Schuppan Detlef (4), Strazzabosco Mario (2)

(1) Dipartimento di Scienze Chirurgiche, Oncologiche e Gastroenterologiche (DiSCOG), Università di Padova, via Giustiniani, 2, 35128 Padova, Tel. +39.328.3337780, E-mail: luca.fabris@unipd.it
(2) Dipartimento di Chirurgia e Medicina Interdisciplinare, Università di Milano-Bicocca, Milano
(3) Department of Medicine and Liver Center, Yale University, New Haven, CT, USA
(4) Division of Gastroenterology Hepatology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

BACKGROUND AND AIMS. Congenital Hepatic Fibrosis (CHF) is caused by mutations in PKHD1, a gene encoding for fibrocystin, a protein of unknown function, expressed in cholangiocyte cilia and centromers. In CHF, biliary dysgenesis is accompanied by severe progressive portal fibrosis and portal hypertension. The mechanisms responsible for portal fibrosis in CHF are unclear. The avß6 integrin mediates local activation of TGFß1 and is expressed by reactive cholangiocytes during cholestasis. To understand the mechanisms of fibrosis in CHF we studied the expression of avß6 integrin and its regulation in Pkhd1-KO mice.
METHODS. In Pkhd1-KO mice we studied, at different ages (1-12 months): a) portal fibrosis (Sirius Red) and portal hypertension (spleen weight/body weight); b) avß6 mRNA and protein expression (RT-PCR, IHC); c) a-SMA and TGFß1 mRNA expression (RT-PCR); d) portal inflammatory infiltrate (IHC for CD45 and FACS analysis of whole liver infiltrate); f) cytokines secretion from cultured monolayers of primary cholangiocytes (Luminex assay); g) cytokine effects on macrophage proliferation (MTS assay) and migration (Boyden chamber); h) TGFß1 and TNFa effects on ß6 integrin mRNA expression by cultured cholangiocytes before and after inhibition of the TGFß receptor type II (TGFßRII); i) TGFß1 effects on collagen type I (COLL1) mRNA expression by cultured cholangiocytes.
RESULTS. Pkhd1-KO mice showed a progressive increase in avß6 integrin expression on biliary cyst epithelia.
Expression of avß6 correlated with portal fibrosis ($r=0.94$, $p<0.02$) and with enrichment of a CD45+ve cell infiltrate in the portal space ($r=0.97$, $p<0.01$). Gene expression of TGFß1 showed a similar age-dependent increase. FACS analysis showed that 50-75% of the CD45+ve cells were macrophages (CD45/CD11b/F4/80+ve). Cultured polarized Pkhd1-KO cholangiocytes secreted from the basolateral side significantly increased levels of KC and IP-10 ($p<0.05$). Both cytokines were able to induce macrophage migration ($p<0.05$). Basal expression of ß6 mRNA by cultured Pkhd1-KO cholangiocytes (0.015±0.002 2dCt) was potently stimulated by two typical macrophage-derived cytokines, such as TGFß1 (0.017±0.002 2dCt, $p<0.05$) and TNFa (0.018±0.003 2dCt, $p<0.05$). Inhibition of TGFßRII completed blunted TGFß1 (0.014±0.003 2dCt, $p<0.05$) but not TNFa effects (0.017±0.001 2-dCt, p=ns) on ß6 mRNA. COLL1 mRNA expression by cultured Pkhd1-KO cholangiocytes (0.0009±0.0003 2dCt) was further and significantly increased after TGFß1 stimulation (0.002±0.0005 2-dCt, $p<0.05$).
CONCLUSIONS Pkhd1-KO cholangiocytes possess increased basolateral secretory functions of chemokines (KC, IP-10) able to orchestrate the macrophage homing to the peribiliary microenvironment. In turn, by releasing TGFß1 and TNFa, macrophages up-regulate avß6 integrin in Pkhd1-KO cholangiocytes. avß6 integrin activates latent TGFß1, further increasing the fibrogenic properties of cholangiocytes.

ABSTRACT N. 228

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BRESIN ELENA**	
Telethon grant N.	**GGP11201**	
Total budget €	**271.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

GENETIC BASIS OF STEROID RESISTANT NEPHROTIC SYNDROME AND IMPLICATIONS FOR THERAPY

Mele Caterina (1), Iatropoulos Paraskevas (1), Maranta Ramona (1), Gamba Sara (1), Curreri Manuela (1), Daina Erica (1), Noris Marina (1), Remuzzi Giuseppe (1,2), Bresin Elena (1)

(1) Centro di Ricerche Cliniche per le Malattie Rare "Aldo e Cele Daccò", Istituto di Ricerche Farmacologiche Mario Negri, Via G.B. Camozzi 3, 24020 Ranica (Bg), Italia, Tel. +39.035.4535327, Fax +39.035.4535373, E-mail: raredis@marionegri.it
(2) Dipartimento di Nefrologia e Dialisi, Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo

Introduction. Nephrotic syndrome is characterized by increased permeability of the glomerular barrier to plasma macromolecules resulting in heavy proteinuria, hypoalbuminemia, and edema. Minimal Change Disease (MCD) and Focal Segmental Glomerulosclerosis (FSGS) are the most common histological lesions. Glucocorticoids are first-line treatment, but despite adequate doses remission is not achieved in 10% of MCD patients and in 60-80% of FSGS patients (Steroid-Resistant Nephrotic Syndrome, SRNS).
These patients are exposed to the risk of life threatening infections, thromboembolic episodes, dyslipidemia, and 40% of them develop end-stage renal disease within 10 years from diagnosis. Genetic studies have shown that mutations in genes encoding proteins important for the podocyte homeostasis and function can cause SRNS (NPHS1, NPHS2, PLCE1, MYO1E, PTPRO, LAMB2, CD2AP, INF2, ACTN4, TRPC6, WT1 and ARHGAP24). Mutations in these genes are found in about 60% of childhood-onset patients and in about 20% of adolescent- or adult-onset patients.
An International Registry dedicated to SRNS was established at the Clinical Research Center for Rare Diseases, aiming to disclose the genetic basis of SRNS and to identify genotype/phenotype correlations for ameliorating counselling and therapy.
Methods. After obtaining informed consent, clinical and laboratory data from patients with SRNS and available unaffected relatives are collected by a dedicated Case Report Form and re-evaluated by a multidisciplinary team with expertise in rare diseases. Biological samples are stored from all participants. Genetic screening by direct sequencing in SRNS genes is performed.
Results. Data and biological samples of 165 patients (101 sporadic cases and 64 familial cases belonging to 29 unrelated families)

have been referred from 15 Italian and 4 international Nephrology Units. Thirty-seven out of 165 present extra-renal manifestations. After clinical data re-evalutation by the multidisciplinary team, 7 patients referred as affected by isolated SRNS received another diagnosis (4 Alport syndrome, 2 Denysh-Drash syndrome and 1 renal-coloboma syndrome).
So far, the genetic cause of the disease has been identified in about 17% of the patients overall. The genes involved are INF2 (9 out of 144 patients analyzed), WT1 (7/91), MYO1E (4/82), PLCE1 (4/53), NPHS2 (1/105), CD2AP (1/50) and PAX2 (2/2). Genetic counselling has been provided when appropriate.
The Registry participates in PodoNet, a European research consortium funded under E-Rare first joint call for podocyte affecting diseases, that follows 1469 patients from 34 different countries.
Conclusions. The establishment of the SRNS registry allowed a better characterization of the patients. A great number of SRNS patients has been recruited increasing the potential to perform epidemiologic, genetic and clinical studies.

ABSTRACT N. 229

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**NORIS MARINA**	
Telethon grant N.	**GGP09075**	
Total budget €	**263.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

COMPLEMENT ABNORMALITIES IN PRIMARY MEMBRANOPROLIFERATIVE GLOMERULONEPHRITIS

Noris Marina (1), Bresin Elena (1), Piras Rossella (1), Sorosina Annalisa (1), Valoti Elisabetta (1), Alberti Marta (1), Bettoni Serena (1), Zito Anna (1), Daina Erica (1), Gamba Sara (1), Donadelli Roberta (1), Gabutti Luca (2), Remuzzi Giuseppe (1,3)

(1) Mario Negri Institute for Pharmacological Research, Clinical Research Center for Rare Diseases Aldo e Cele Daccò, via Camozzi 3, 24020 Ranica (BG), Italy, Tel. 035.4535362, Fax +39.035.4535377, E-mail: marina.noris@marionegri.it
(2) Department of Internal Medicine and Division of Nephrology, Ospedale la Carità, Locarno, Switzerland
(3) Department of Nephrology and Dialysis, Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo, Italy

Membranoproliferative glomerulonephritis (MPGN) is a rare kidney disease and accounts for about 4-7% of primary renal causes of nephrotic syndrome. It occurs mainly in children and young adults and frequently progresses to end-stage renal disease. The distinctive feature of MPGN is hypocomplementemia. It is histologically characterized by diffuse mesangial expansion due to endocapillary proliferation, increased mesangial matrix and thickened capillary walls. Distinct types of MPGN have been described based on immunofluorescence staining, ultrastructural appearance, and complement profiles: type I MPGN, the most common variant; type II MPGN; type III MPGN and C3 glomerulonephritis (C3GN).
In 2006 an Italian Registry of Primary Membranoproliferative Glomerulonephritis has been established at the Clinical Research Center for Rare Diseases, with the following aims: to collect clinical data of MPGN patients, to study the genetic and biochemical abnormalities and to provide the best therapeutic approach for each patient.
More than 130 cases of MPGN (age 1-80 years) have been referred to the Registry from 20 Units of Nephrology: 34 patients with type I MPGN, 28 with type II, 4 with type III, 18 with C3GN, 35 with undefined MPGN, 13 with overlapping MPGN and thrombotic microangiopathy. Persistently low C3 levels were recorded in most patients, including all MPGN types and C3 nephritic factor activity was positive in 40% of them.
Mutations in genes known to be involved in atypical Hemolytic Uremic Syndrome, encoding Factor H (CFH, 5.3%), Factor I (CFI, 3.1%), Membrane Cofactor Protein (MCP, 1.3%) and Complement factor 3 (C3, 1.5%) have been identified in a small number of MPGN patients (10.6%).
Ongoing studies are focused on functional consequences of complement genetic abnormalities and in searching for new gene mutations/variants that may be involved in the predisposition to MPGN because in most patients the cause of the disease is unknown. Candidate genes are those encoding proteins of the complement system due to the clear-cut evidence that MPGN is a disease of complement hyperactivation.
High levels (>1000 ng/ml) and moderately high levels (500 to 999 ng/ml) of the terminal complement complex (sC5b-9: nv 127-303 ng/ml) were found respectively in 25% and 13% of the evaluated patients. This marker of complement activation should be potentially used to identify patients who could benefit from treatment with Eculizumab, a humanized monoclonal antibody that binds to complement factor C5 and inhibits the generation of the terminal lytic complement complex. In this regard, among patients with MPGN who have been recently treated with Eculizumab, a good clinical response was observed only in those patients (two of them from our Registry) who showed high levels of sC5b-9 before treatment. These data suggest that Eculizumab could be an effective treatment in the subgroup of MPGN patients with hyperactivation of the terminal pathway of complement.

ABSTRACT N. 230

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**LUPETTI PIETRO**	
Telethon grant N.	**GGP07269**	
Total budget €	**208.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2007**

HIGH RESOLUTION STRUCTURAL STUDY OF THE IN SITU AND PURIFIED INTRAFLAGELLAR TRANSPORT (IFT) COMPLEX: KEY MOLECULAR MACHINERY ESSENTIAL FOR CILIOGENESIS AND THE CELLULAR BASIS FOR POLYCYSTIC KIDNEY DISEASE

Vannuccini Elisa (1), Cantele Francesca (2), Gentile Mariangela (1), Dini Daniele (1), Lanzavecchia Salvatore (2), Paccagnini Eugenio (1), Lupetti Pietro (1)

(1) Dip. Scienze della Vita, Università degli Studi di Siena, Via Aldo Moro 2, 53100 Siena, Tel. 0577.234402, Fax 0577.234476, E-mail: pietro.lupetti@unisi.it
(2) Dip. di Chimica Strutturale, Università di Milano, Milano

Polycystic Kidney disease (PKD) is one of the most common inherited diseases. It has been shown that the several PKD variants are due to defects in the primary cilium emanating from each of the cell of the kidney tubules. PKD is caused by defects in a process called Intraflagellar Transport (IFT) a bi-directional movement of polypeptide particles between the ciliary membrane and the external microtubular doublets. IFT trains are required for the assembly and maintenance, as well as the sensory function, of all primary (non-motile) and motile cilia. The biochemical and molecular biology analyses of IFT particles have provided a fairly good amount of information while only little was known about the ultrastructure of IFT complexes until we published in 2009 the first high-resolution 3D ultrastructural analysis of long IFT train particles in situ, using electron-tomographic analysis of flagellar sections from flat-embedded Chlamydomonas cells. Analyzing in depth flagella from vegetative cells, we identified two different kinds of trains differing for their overall length and ultrastructure: long narrow trains and short compact trains, each with a characteristic inner repeat. In order to understand if the structural differences among the two types of IFT trains could be related to different functions, we quantified the number of long and short trains in regenerating flagella of wt 137c cells and in reabsorbing flagella of temperature sensitive mutant pf1-fla10-1 cells that, when incubated at 32°C are lacking a subunit of the retrograde IFT molecular motor. The first evidences are that the long trains seems to have a predominant role on the anterograde transport while the amount of the short trains per flagellum seems not related to the flagellar regeneration and reabsorption. Further analyses are necessary to understand this system that is more complex than predicted, as it is shown in other recent studies. Other recent data are coming from the three dimensional reconstruction of the short complexes obtained by the new strategy of alignment developed ad hoc in collaboration with the research group of prof. Salvatore Lanzavecchia, at the University of Milan. These trains show two links to the B subtubule of microtubular doublets. Such links are connected to a basal electron dense domain from which major densities are visible in the core domain and connected to flagellar membrane. The short trains present also an unexpected repeat on top of their longitudinal core between the microtubular doublet and the flagellar membrane. Another aspect that we are deling with is the ultrastructural analysis of the isolated IFT particles. We succeeded in developing a new extraction protocol that allowed us to obtain native strings of isolated IFT trains. Immugold labelling of such material demonstrated for the first time that both A and B IFT subcomplexes are present in IFT trains. In addition to these evidences, our brand new protocol of isolation will provide us with suitable material for further structural examination of complex A and B associations within the train by use of high res-

olution imaging procedures. We are also planning to extend our structural analyses to the study of the conformational change between long and short IFT trains occurring at the flagellar tip and at the transition zone, the selective gate controlling trafficking of flagellar components between the cytoplasm and the flagellar compartment.

ABSTRACT N. 231

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GHIGGERI GIAN MARCO**	
Telethon grant N.	**GGP08050**	
Total budget €	**355.700**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2008**

GENETIC DETERMINANTS OF NON-SYNDROMIC RENAL HYPODYSPLASIA AND RELATED PHENOTYPES

Sanna-Cherchi Simone (3), Bodria Monica (1), Scolari Francesco (4), Caridi Gianluca (1), Carrea Alba (1), Allegri Landino (2), Gharavi Ali G. (3), Ghiggeri Gian Marco (1)

(1) UOC.di Nefrologia, Dialisi e Trapianto, Laboratorio di Fisiopatologia dell'Uremia, Istituto Giannina Gaslini, Largo G. Gaslini, 5 16148 Genova, Italy, Tel. +39.010.380742, Fax +39.010.395214, E-mail: GMarcoGhiggeri@ospedale-gaslini.ge.it
(2) Dipartimento di Medicina Clinica, Nefrologia e Scienze della Vita, Università di Parma, Parma, Italy
(3) Division of Nephrology, Columbia University College of Physicians and Surgeons, New York, NY USA
(4) Divisione di Nefrologia e Dialisi, Ospedale di Montichiari (BS), Università di Brescia, Italy

Urogenital malformations are present in more than 20% of overall birth defects. They overall identify a clinical conditions known as Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) that accounts for 40-50% of pediatric and 7% of adult end-stage kidney failure worldwide, with significant impact on renal survival in adulthood. Advancement in accurate diagnosis, prevention and treatment of these diseases is hampered by many factors including a lack of well-validated methods to access disease progression, by a lack of insight into the genetics of the disorders, absence of well formulated and tested definitions of the disease and its possible clinical subcategories, by a lack of rigorous epidemiological data and by an incomplete understanding of the pathophysiology of many of these diseases and disorders.
We applied novel genomic approaches to look for rare variants with large effect in patients with renal hypodysplasia, a severe congenital renal malformation. We used high density arrays to identify rare submicroscopic structural variants (deletions/duplication) in sporadic patients, and next-generation sequencing to identify segregating point mutations is familial forms of disease. We detected 72 distinct known or novel genomic disorders in up to 16.6% of patients (10.5% known disorders, 6.1% rare or novel disorders) a part of which are associated with risk of neuropsychiatric diseases (DiGeorge/velocardiofacial syndrome, chromosome 1q21 deletion, the 2q37 deletion, the 17p11.2 duplication).
By next generation sequencing on familial CAKUT we identify pathogenic mutations in VUR1 gene , a novel mediator of FGF signaling, in up to 3% of patients with urinary tract malformations.
Altogether, novel genomic approaches allowed us to identify pathogenic mutations and perform molecular diagnosis in a large proportion of patients, with implications on genetic counselling and individualization of treatments.

ABSTRACT N. 232

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TONIOLO DANIELA**	
Telethon grant N.	**GGP09126**	
Total budget €	**574.700**	
Centres: **4**	*Duration (yrs):* **3**	*Starting year:* **2009**

DISSECTING THE GENETIC BASIS AND MOLECULAR MECHANISMS OF PREMATURE OVARIAN FAILURE

Toniolo Daniela (1,4), Persani Luca (2), Marozzi Anna (3), Rossetti Raffaella (2), Caslini Cecilia (3), Bione Silvia (4), Corre Tanguy (1), Sala Cinzia (1), Barbieri Caterina (1)

(1) San Raffaele Scientific Institute, Milano, via Olgettina 58, 20132 Milano, Tel. +390226434764, Fax +390226434767, E-mail: daniela.toniolo@hsr.it
(2) Division of Endocrine and Metabolic Diseases & Lab. of Endocrine and Metabolic Research, Ospedale San Luca, IRCCS Istituto Auxologico Italiano, Milano
(3) Dipartimento di Biologia e Genetica per le Scienze Mediche, Università di Milano
(4) Institute of Molecular Genetics, CNR, Pavia

Premature Ovarian Failure (POF) is a heterogeneous disorder with a relevant genetic component. It is characterized by cessation of menses before 40 years of age, elevated levels of serum gonadotropins and sex hormones deficiency. The project aim is twofold:
i) Identification of genes involved in the pathogenesis of POF and ii) Investigation of the mechanisms of action of two genes, DIAPH2, BMP15 and of the FMR1 premutated allele.
The coordinator and Partner 2 have been involved in Genome Wide association Analysis (GWAS) of the POF samples collected in previous years. The Val Borbera and the POF NIDO cohort became part of the large international consortium (the Reprogen Consortium), whose aim is to study the female reproductive life span. GWAS to identify genes for age of menopause, EM and POF were completed and 17 loci were identified: altogether they increase the risk of POF well above that of smoking. Functional studies have demonstrated a role for the DIAPH2 gene in granulosa cells remodeling following FSH stimulation.
Partner 1 performed a phylogenetic analysis on the protein sequence of BMP15 in different species and showed a significant increase in the BMP15-dependent signaling for some of the selected variants. The analysis of the transcripts by Real Time PCR did not show a direct correlation between the expression levels and the increased biological activity of the three variant forms. These preliminary data may support the hypothesis that also mutations leading to an increased BMP15 protein synthesis or an overstimulation BMP15-dependent of the granulosa cells could be related to POF, possibly affecting the delicate equilibrium between intra-follicle factors and the hypothalamus-pituitary-ovary axis. Further candidate genes with a possible role for the pathogenesis of POF have been identified from the analysis of the transcriptome induced by BMP15 in primary cultures of granulosa cells (GCs). BMP15 induces the expression of genes important for the regulation of BMPs signaling pathway already two hours after stimulation (SMAD6, -7, ID1, -2, -3, BAMBI) and their upregulation is maintained even after 6h. BMP15 can also inhibit the expression of genes important in the ovarian physiology, both early (such as: FST) and later (TGFB3R, STAR, LHCGR, SMAD3, ADAMTS1, FST) as well as of genes relevant for the regulation of anti-apoptotic and proliferative processes (BCOR , BCL6).
Partner 4 utilized an in vitro model of premutated ovarian granulosa cell and showed several proteins able to directly interact with riboCGG repeats. In granulosa cells expressing expanded CGG-repeat RNA a transcriptional up-regulation of the stress response genes CRYAB, HSP27, and a parallel increase of the protein levels was observed; in particular the significant increase in accumulation of CRYAB in the RIPA-insoluble (SDS-soluble) protein fraction might reflect changes in protein translocation into the nuclei, reflecting an early response to the cellular stress imposed by the expression of the expanded repeat. Furthermore the viability of granulosa expressing the expanded CGG repeat is significantly reduced suggesting a toxic role of CGG-repeat mRNA is itself in the ovaries.

ABSTRACT N. 233

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**ROSSI ANTONIO**	
Telethon grant N.	**GGP11079**	
Total budget €	**174.200**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

THE FUNCTIONAL ROLE OF THE CALCIUM ACTIVATED NUCLEOTIDASE 1 (CANT1) GENE IN THE SKELETON: AN IN VIVO STUDY WITH A MOUSE MODEL OF DESBUQUOIS DYSPLASIA

Monti Luca (1), De Leonardis Fabio (1), Forlino Antonella (1), Tenni Ruggero (1), Huber Celine (2), Nizon Mathilde (2), Cormier-Daire Valerie (2), Rossi Antonio (1)

(1) Università di Pavia, Dipartimento di Medicina Molecolare, sez Biochimica, via Taramelli 3/B, 27100 Pavia, Tel.+39.0382.987229; Fax +39.0382.423108; E-mail: antrossi@unipv.it
(2) University Paris Descartes, Dept. Genetics and INSERM U781, Hôpital Necker Enfants Malades, Paris, France

Desbuquois dysplasia (DD) is an autosomal recessive chondrodysplasia characterized by antenatal and postnatal growth retardation, multiple dislocations and advanced carpal ossification. Two forms of DD have been described on the basis of the presence (type 1) or absence (type 2) of characteristic hand deformities. Studying DD type 1 families, mutations in the Calcium-Activated Nucleotidase 1 gene (CANT1) have been identified (Huber C et al (2009) Am J Med Genet, 85, 706-710 and Nizon M et al (2012) Hum Mut, 33, 1261-66).
CANT1 is a calcium activated nucleotidase that preferentially hydrolyzes UDP; two different forms of the enzyme have been characterized, but their functions remain to be elucidated: i) a membrane bound form in the endoplasmic reticulum and Golgi. The subcellular location and the substrate preference (UDP) suggest CANT1 involvement in protein glycosylation and protein quality control; ii) a soluble secreted form that can be involved in pyrimidinergic signaling by modulating the availability of extracellular UDP to P2Y receptors, a group of G protein coupled receptors.
DD shares phenotypic features with other chondrodysplasias characterized by defects in cartilage proteoglycan metabolism, the main cartilage glycoproteins. To test whether CANT1 deficiency interfere with the availability of UDP-sugars needed for proteoglycan synthesis, fibroblasts from two DD patients homozygous for the p.R300H and p.P245RfsX3 mutations respectively, and four controls were double labeled with [35S]sulfate and [3H]glucosamine. In the patient cells glycosaminoglycan (GAG) synthesis was almost normal under basal conditions, but significant reduced GAG synthesis was observed in presence of beta-D-xyloside, a compound which enhances synthesis and secretion of chondroitin and dermatan sulfate chains acting as a chain initiator. Furthermore gel filtration chromatography on Superose 6 of GAGs released from newly synthesized proteoglycans after beta-elimination demonstrated that GAG chains were shorter compared to the controls. Hyaluronic acid synthesis which occurs in the plasma membrane, a different compartment from proteoglycans, was within normal values supporting the involvement of CANT1 in the ER/Golgi compartment. These data suggest that CANT1 plays a role in proteoglycan metabolism (Nizon M et al (2012) Hum Mut, 33, 1261-66). Thus, to define the physiological function of CANT1 and its role in the etiology of DD we are generating a CANT1 knock-in mouse reproducing a mutation already detected at the homozygous state in several patients affected by DD type1. All studies will be performed on the mouse model of DD we will generate since they cannot be done in human patients for ethical reasons. These studies are designed to yield a deeper understanding of the function of CANT1 and of the pathogenesis of inherited forms of osteoarthropathy, so that effective therapies can be developed.

ABSTRACT N. 234

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GENNARI LUIGI**	
Telethon grant N.	**GGP11119**	
Total budget €	**125.200**	
Centres: **2**	*Duration (yrs):* **2**	*Starting year:* **2011**

GENETICS AND PHARMACOGENETICS OF PAGET'S DISEASE OF BONE

Gianfrancesco Fernando (2), Formicola Daniela (2), Esposito Teresa (2), Rendina Domenico (1), Merlotti Daniela (1), Magliocca Sara (2), Muscariello Riccardo (1), Strazzullo Pasquale (3), Nuti Ranuccio (1), Gennari Luigi (1)

(1) Department of Internal Medicine, Endocrine-Metabolic Sciences and Biochemistry, University of Siena, Policlinico Santa Maria alle Scotte, Viale Bracci, 53100 Siena, Tel. 0577.585364, Fax 0577.233446, E-mail: gennari@unisi.it
(2) Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", CNR, Naples, Italy

Paget's disease of bone (PDB) is an invalidating disease with a strong genetic component. Despite the recent advances (including the identification of SQSTM1 mutations in up to 40% of families), the pathogenesis of PDB remain in part unknown as well as the effects of SQSTM1 mutation on the phenotype and the response to treatment.
The main objectives of the project were the following: 1) the identification of a new gene causing PDB and giant cell tumor through next-generation sequencing of a large Italian pedigree with 14 affected cases; and 2) to perform an extended pharmacogenetic study on the effects of SQSTM1 gene mutations and other disease-modifying genes on the response to bisphosphonate treatment.
Aim 1: Affected and unaffected cases from the selected pedigree were further characterized from the clinical point of view. All the affected members had polyostotic PDB, but the 4 subjects developing giant cell tumors showed an increased disease severity with a reduced clinical response to bisphosphonate treatment and an increased prevalence of bone pain, deformities, and fractures. Together with an increased occurrence of common pagetic complications, affected patients of this pedigree also evidenced a 5-fold higher prevalence of coronary artery disease with respect to either the unaffected family members or a comparative cohort of 150 unrelated PDB cases from the same geographical area. Genomic DNA samples from 6 affected and 2 unaffected members belonging to this pedigree were assayed using the NimbleGen SeqCap EZ Exome™ capture kits (Roche) and resultant fragments sequenced with one lane per sample on an Illumina GAIIx (Illumina, San Diego, CA) with 90 bp paired-end reads. More than 50,000,000 paired-end reads (Average Coverage 50X) for each member were obtained and aligned to the human reference genome sequence (GRCh37/hg19) with MAQ7 and NextGENe software v2.00 with sequence condensation by consolidation (SoftGenetics, State College, PA). This approach resulted in more than 88% of target exomes being covered by ten reads or more. We matched data from all individuals to obtain shared variants. 57 non-synonymous coding variants were shared between affected samples and were not present in unaffected samples. Only 32 (66%) of these variants were confirmed by direct sequencing but 24 of them were present in 1000 Genome database. Of the 8 novel variants only 3 showed complete segregation with the disease in our family. Interestingly, these 3 variants are located on the chromosome 1 where our previous linkage analysis identified a putative region segregating with the disorder.
Aim 2: More than 800 PDB cases have been screened for SQSTM1 mutation and other polymorphic variants of disease modifying genes or genes potentially associated with the response to bisphosphonate treatment for analysis of retrospective and prospective data. Association analyses will be performed during the second year, at the end of the follow-up observation period.

ABSTRACT N. 235

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MATTEVI ANDREA**	
Telethon grant N.	**GGP12007**	
Total budget €	**221.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

PEROXISOMAL ENZYME DEFICIENCIES IN RHIZOMELIC CHONDRODYSPLASIA PUNCTATA: BIOCHEMISTRY AND THERAPEUTIC AVENUES

Mattevi Andrea, Nenci Simone, Piano Valentina

Dipartimento di Biologia e Biotecnologie "L. Spallanzani", Università degli Studi di Pavia, via Ferrata 9, 27100 Pavia, Tel. 0382.985534, Fax 0382.528496, E-mail: andrea.mattevi@unipv.it

The importance of peroxisomes for normal mammalian development and growth is underlined by the existence of a heterogeneous group of inherited diseases, the peroxisomal disorders. Among the single peroxisomal enzyme deficiencies, Rhizomelic Chondrodysplasia Punctata affects about 1 in 100,000 individuals, causing skeletal malformations, serious mental retardation and infant death.
RCDP shows defects in the synthesis of a particular class of membrane phospholipids, characterised by an ether bond (rather than an ester bond) connecting the alkyl chain to the sn-1 carbon of the glycerol moiety. The most abundant members of this class are plasmalogens. In RCDP, plasmalogens deficiency is directly caused by mutations in two peroxisomal enzymes performing the crucial step in ether-phospholipids synthesis: dihydroxyacetone phosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetone phosphate synthase (ADPS). The first generates the acyl-DHAP molecule that is subsequently used by ADPS to catalyse the replacement of the acyl-group with a long-chain fatty alcohol, generating the precursor of all ether-phospholipids, alkyl-DHAP (Ronald JA, et al. Annu Rev Biochem. 2006;75:295-332).
Our work has been focused on the understanding of the structural and functional features of the peroxisomal flavoprotein ADPS, in order to exploit the phenotype/genotype correlation of the mutations associated with RCDP.
For this purpose, the recombinant ADPS enzyme from C. porcellus (95% of identity with human ADPS) has been expressed and purified to perform crystallisation trials, that leaded to the solution of the crystal structure of the enzyme.
We produced and studied five mutants found in RCDP patients,

which affect either the cofactor or the substrate binding. The point emerging from these experiments is that all mutations fully inactivate the enzyme, supporting the notion that the disease-causing mutations knock-out de novo biosynthesis of ether phospholipids.
The important information collected allowed us to hypothesize a non-redox reaction mechanism, unusual for flavoproteins, which has been extensively investigated through spectrophotometric and mass spectroscopy assays and resulted to converge on the formation of a covalent adduct with the substrate to enable acyl-alkyl exchange (Nenci S, et al. PNAS, 2012, Nov 13;109(46):18791-6).
To complete the picture of the situation, our attention has been extend to the enzyme DHAPAT, which is believed to directly interact and form a heterotrimeric complex with ADPS in vivo. For this reason, the characterisation of DHAPAT and the reproduction of the complex in vitro could be determinant to deeply understand the biochemical and structural features of the interaction underlying the crucial step of ether-phospholipids biosynthesis.

ABSTRACT N. 236

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BIANCO PAOLO**	
Telethon grant N.	**GGP09227**	
Total budget €	**262.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

FIBROUS DYSPLASIA OF BONE - TRANSGENIC MODELS OF DISEASES, AND MODELS OF THERAPY

Riminucci Mara (1), Saggio Isabella (2), Remoli Cristina (1), Cersosimo Stefania (1), Sacchetti Benedetto (1), Spica Emanuela (1), Astrologo Letizia (1), Gehron Robey Pamela (3), Davis Graham (4), Holmbeck Kenn (3), Boyde Alan (4), Piersanti Stefania (2), Comite Paola (1), Michienzi Stefano (1), Bianco Paolo (1)

(1) Dipartimento di Medicina Molecolare, Università La Sapienza, Roma, Viale Regina Elena 324, 00161 Roma, Italy, Tel. + 39.06.4441049, Fax +39.06.4461972, E-mail: paolo.bianco@uniroma1.it
(2) Dipartimento di Genetica e Biologia Molecolare, Università La Sapienza, Roma, Italy
(3) NIDCR, NIH, Bethesda MD, USA
(4) Queen Mary's, University of London, UK

Fibrous Dysplasia (OMIM174800) is a crippling skeletal disease caused by activating mutations in the Gsa gene. Animal models of FD have been woefully needed for understanding disease mechanisms, and for developing and testing mechanistic therapeutic approaches. We generated and extensively analyzed multiple lines of transgenic mice that express the disease causing mutation, GsaR201C, either constitutively or as targeted to specific cell types. We have now obtained a detailed, comprehensive, and entirely novel appraisal of the disease biology, and specific prospects for therapy. First, in the mouse, constitutive expression of GsaR201C is compatible with germline transmission, resulting in a replica of the human disease, precise but inherited, and independent of mosaicism; it does not affect development, skeletogenesis, or prenatal bone formation. Second, targeting the mutation to osteoblasts generates a marked high bone mass phenotype (mediated by downregulation of SOST), which phenocopies human disorders caused by loss-of-function mutations in the SOST gene (Van Buchem's disease, Sclerosteosis), but not FD. All of the defining tissue changes and direct morbidity factors in FD bone (osteolysis, fracture, osteomalacia, marrow fibrosis) reflect the effects of the mutation on compartments of the stromal/osteogenic lineage other than osteoblasts. Third, bone marrow fat cells are the unsuspected culprits of abnormal bone formation in FD. GsaR201C causes upregulation of UCP-1 and thermogenic lipolysis in marrow fat, WAT, and BAT, mimicking the effects of ß-adrenergic stimulation in 'beige' fat. In bone, conversion of mutated fat cells into dysplastic, fat-derived, osteoblasts ensues. Ectopic expression of the mineralization inhibitor, MGP, is the hallmark of such unique osteoblasts, and the cause of osteomalacia and deformity in FD bone. MGP is not expressed in osteoblasts with targeted GsaR201C expression; MGP-producing osteoblasts only arise from mutated precursors. Fourth, GsaR201C expression in ADRB2-enriched perivascular osteoprogenitors, but not in PTH1R-enriched mature bone cells, causes overexpression of RANKL in osteoprogenitors, but not in mature bone cells; mutated osteoprogenitors cue excess osteoclastogenesis, which causes osteolysis and fracture in FD bone. In WT osteoprogenitors, this effect of the mutation is mimicked by stimulation of ADRB2 (ISO), but not of PTH1R (PTH). Fifth, distinct changes and mechanisms mark distinct temporal phases in the natural history of the disease, with distinct windows for distinct types of treatment. Sixth, the Gsa-dependent, key mechanisms in FD (RANKL, MGP, beige marrow fat) do operate in human FD, and can be reproduced in human osteoprogenitors (transduced with the same constructs used to generate FD mice). FD is a dysregulation of the neural/ß-adrenergic control of the osteogenic lineage as a system. All the novel disease mechanisms we identified are druggable.

ABSTRACT N. 237

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**SOBACCHI CRISTINA**	
Telethon grant N.	**GGP12178**	
Total budget €	**123.000**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2012**

RANKL ADMINISTRATION AND MESENCHYMAL STEM CELL TRANSPLANTATION AS THERAPEUTIC APPROACHES TO RANKL-DEPENDENT OSTEOPETROSIS

Lo Iacono Nadia (1,2), Marrella Veronica (1,2), Ficara Francesca (1,2), Poliani Pietro L (3), Traggiai Elisabetta (4), Kostenuik Paul (5), Blair Harry (6), Villa Anna (1,2), Sobacchi Cristina (1,2)

(1) CNR-IRGB, Milan Unit, via Manzoni 113, 20089 Rozzano, Milan, Italy, Tel. +39.02.82245164, Fax +39.02.82245191, E-mail: cristina.sobacchi@humanitasresearch.it
(2) Humanitas Clinical and Research Center, Rozzano, Milan, Italy
(3) University of Brescia, Brescia, Italy
(4) Istituto G. Gaslini IRCCS, Genoa, Italy
(5) Metabolic Disorders Research Unit, Amgen Inc, Thousand Oaks, USA
(6) University of Pittsburgh, Pittsburgh, USA

Autosomal Recessive Osteopetrosis (ARO) is a rare genetic bone disease presenting early in life with extreme sclerosis of the skeleton, causing hematologic and neurologic defects. ARO is often lethal because of anaemia and secondary infections, and haematopoietic stem cell transplantation (HSCT) remains the only therapeutic option. So far, the RANKL-dependent ARO is unique because the defect is not intrinsic to the hematopoietic osteoclast lineage and cannot be rescued by HSCT. In order to identify an effective therapy for this subset of patients, we tested the effects of the subcutaneous administration of 1 mg/kg soluble RANKL to Rankl-/- mice, which closely resemble the human condition. Treatment was performed since neonatal age every 48 hours.
After 1 month, the bone phenotype of treated Rankl-/- mice was rescued and secondary defects related to the disease, such as extramedullary haematopoiesis, were substantially improved, with a significant increase in bone marrow cellularity and an important amelioration of splenic structure and cell populations. This treatment regimen appeared to be well tolerated, without obvious adverse effects over the 1 month duration of follow-up.
On the other hand, 3 month administration of 1 mg/kg RANKL to Rankl-/- mice overstimulated bone resorption and caused a marked reduction of the mineral content, enlargement of lymphoid aggregates, formation of pulmonary exudates with reduction of lung function and the death for all treated mice.
Overall, we demonstrated that short-term treatment with RANKL rescued the bone defect in Rankl-/- mice without major side effects; the safety issues raised by overtreatment in mice highlighted the importance of carefully monitoring this therapy in patients.
In spite of our encouraging results, due to the limited number of individuals which would benefit from this therapy as well as to the costs and complex rules regulating the administration of unapproved drugs, we were unable to obtain GMP RANKL for testing in humans. Therefore, taking advantage of our expertise on cell therapy in murine models (Frattini et al, 2005; Panaroni et al, 2009; Tondelli et al, 2009) and of the positive results already reported with Mesenchymal Stem Cell Transplantation (MSCT) in patients affected by Osteogenesis Imperfecta (Horwitz et al, 2002; Le Blanc et al, 2005), we decided to evaluate also this approach in Rankl-/- mice. If our preclinical results are encouraging, we will easily move to the clinical level, thanks to the collaboration with the Stem Cell Factory of Ospedale San Gerardo (Monza).
So far, we have produced 2 lines of BM-derived MSCs, obtained from C57BL/6J GFP+ and C57BL/6J 6RFP+ mice, respectively. These cells will be used for neonatal and in utero MSCT; we will study the effects on bone, lymphoid organs (thymus, spleen, lymph nodes) and other organs potentially affected by the treatment (i.e. lung, kidney, liver, heart and brain).

ABSTRACT N. 238

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TETI ANNA MARIA**	
Telethon grant N.	**GGP09018**	
Total budget €	**200.100**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

NEW THERAPEUTIC APPROACHES TO OSTEOPETROSIS

Cappariello Alfredo (2), Del Fattore Andrea (1,2), Rucci Nadia (1), Teti Anna Maria (1)

(1) Dipartimento di Scienze Cliniche Applicate e Biotecnologie, Via Vetoio Coppito 2, 67100 L'Aquila, Tel. 0862.433513; Fax 0862.433523, E-mail: teti@univaq.it
(2) Ospedale Pediatrico Bambino Gesù, Roma

The aim of this project is to set up experimental therapies to cure two forms of osteopetrosis, a rare genetic disease impairing osteoclast bone resorption and leading to dense but fragile bones, hematologic and neural failures. The first autosomal recessive form is due to loss-of-function mutations of the TNFSF11 gene, causing deficiency of the pro-osteoclastogenic cytokine RANKL and lack of osteoclast formation. We designed a diffusion chamber, which isolates cells but allows flow of molecules, carrying 3D hydroxyapatite scaffolds on the surface of which we adsorbed the catalytic domain of the RANKL-shedding enzyme MMP-14 to promote release of the membrane-bound RANKL active domain. RANKL-producing cells, namely mouse primary calvarial osteoblasts or osteoblast-like MC3T3 cells stably transfected with RANKL, were implanted on scaffold and sealed in the diffusion chambers, allowing the release of detectable amounts of soluble RANKL in conditioned media. These devices were inserted in the abdomen of 21 days old RANKL knockout (KO) mice which were sacrificed after 30 days from implant. Histological sections of control tibias were negative for the histochemical detection of the osteoclast marker TRAcP, consistent with the lack of the osteoclast lineage. In contrast, tibias excided from implanted mice showed the appearance of TRAcP-positive cells both on the bone surface and within the bone marrow, suggesting the induction of osteoclastogenesis. Optimization of the procedure is currently in progress to establish conditions compatible with the improvement of the phenotype.
The second autosomal dominant form of osteopetrosis is caused by single allele missense mutations of the CLC7 gene encoding the homodimeric subunits of the type 7 Cl-/H+ antiporter, indispensible for bone resorption. We silenced the mutant allele by treatment of mutant CLC7-transfected cells with highly specific siRNAs, which did not affect the normal mRNA both in vitro and in xenografts. We then administered siRNA against the normal allele to WT mice, showing its ability to dose- and time-dependently downregulate CLC7 transcriptional expression in tibias and other organs. WT mice were also treated with siRNAs against the mutant CLC7 which proved ineffective in reducing the WT mRNA, thus confirming their high specificity. To test the siRNA against the mutant allele in vivo, we have generated a clc7-p.G213R knock-in (KI) mouse model carrying the homolog (p.G215R) of the most frequent human CLC7 mutation. Similar to patients, homozygous mice show a severe autosomal recessive osteopetrosis, while heterozygous mice exhibit milder symptoms consistent with the autosomal dominant form. siRNAs against the clc7-p.G213R allele were tested in vitro to isolate the best performing sequences that will now be injected in the heterozygous clc7-p.G213R KI mice to examine their efficacy, specificity and ability to improve the osteopetrotic phenotype.

ABSTRACT N. 239

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**FIUMARA FERDINANDO**	
Telethon grant N.	**GGP11223**	
Total budget €	**147.300**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

POLYALANINE COILED COILS REGULATE PROTEIN AGGREGATION, FUNCTION, AND GENETIC DISEASE-RELATED DYSFUNCTION

Pelassa Ilaria (1), Corà Davide (2), Fiumara Ferdinando (1)

(1) Department of Neuroscience, Università degli Studi di Torino, Corso Raffaello 30, 10125 Torino, Tel. 011.6708486, E-mail: ferdinando.fiumara@unito.it
(2) Center for Molecular Systems Biology, Università degli Studi di Torino, Torino

The pathological expansion of DNA triplet repeats encoding for glutamine (Q) or alanine (A) can cause severe diseases in humans, including Huntington's disease and cleidocranial dysplasia. PolyQ and polyA repeats are physiologically present in many proteins, at times in association, but their excessive elongation in triplet expansion diseases causes protein aggregation and dysfunction, leading to cell damage or death. The structural and molecular mechanisms by which expanded polyQ and polyA traits cause these pathological phenomena are only partially understood.
We have recently found that polyQ repeats participate in the formation of alpha-helical coiled coil (CC) supersecondary structures that have a critical role in triggering the aggregation and toxicity of polyQ-expanded proteins. The fact that polyA repeats often co-occur with polyQ repeats, even in proteins linked to expansion diseases, prompted us to investigate the possibility that polyA repeats may also form CC structures that may be important for physiological protein function, and for pathological protein aggregation and dysfunction upon polyA expansion.
To address these issues, we first performed bioinformatics screenings of human and non-human proteomes to assess the occurrence and co-occurrence of polyA and polyQ repeats, and their degree of association with CC domains. We have found in all the proteomes tested a significant association of A and Q repeats, and a significant overlap of polyA stretches with CC domains.
To verify experimentally that polyA repeats are indeed prone to form CC structures, we synthesized peptides containing polyA stretches of either physiological or pathological length. Circular dichroism experiments revealed that polyA stretches have propensity to form alpha-helical CCs, and that longer alanine repeats confer higher stability to these structures. Chemical cross-linking showed that the presence of longer polyA repeats favors the formation of higher-order multimers that may drive aggregation in vivo.
To test the relevance of polyA CCs for in vivo aggregation, function, and dysfunction of polyA proteins, we performed structure-guided mutagenesis of the RUNX2 protein, a transcription factor containing CC-prone polyQ and polyA stretches, that causes cleidocranial dysplasia upon polyA expansion. We generated mutants with enhanced or reduced CC stability of either wild type (wt) or polyA-expanded RUNX2. Overexpression of these mutants in cell lines showed that polyA CC stability is a key regulator of the physiological function of wt RUNX2, and of the pathological aggregation and dysfunction of polyA-expanded RUNX2, in cooperation with the flanking polyQ CC.
These findings indicate that polyA CC structures have a fundamental role in the aggregation, function, and dysfunction of polyA proteins, and support a unitary CC model for the protein structural dynamics underlying polyQ- and polyA-expansion diseases.

ABSTRACT N. 240

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MELINO GENNARO**	
Telethon grant N.	**GGP09133**	
Total budget €	**223.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

SENSORINEURAL DEAFNESS IN EEC SYNDROME PATIENTS: INVOLVEMENT OF P63 APOPTOSIS/DIFFERENTIATION PATHWAY IN COCHLEAR NEUROEPITHELIUM

Alessandro Terrinoni (1), Valeria Serra (1), Ernesto Bruno (1), Andreas Strasser (2,3), Elizabeth Valente (2), Hans van Bokhoven (4), Xin Lu (5), Richard A. Knight (6), Gerry Melino (1,6)

(1) Biochemistry Laboratory IDI-IRCCS, c/o Department of Experimental Medicine and Surgery, University of Rome "Tor Vergata", via Montpellier 1, 00133 Rome, Italy, Tel. +39.06.72596976, Fax +39.06.72596977; E-mail: melino@uniroma2.it
(2) The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia
(3) Department of Medical Biology, Melbourne University, Parkville, Victoria, Australia
(4) Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
(5) Nuffield Department of Clinical Medicine, Ludwig Institute for Cancer Research, University of Oxford, Oxford, UK
(6) Medical Research Council, Toxicology Unit, Hodgkin Building, Leicester University, Leicester, UK

The Ectodermal dysplasia and Cleft lip/palate (EEC) syndrome is clinically characterised by ectodermal dysplasia affecting skin, hair, nails and teeth, and facial clefts. It is well known that heterozygous

mutations in the p63 gene have been identified in EEC and EEC-like patients.
p63 it is an important protein for the epithelial formation and during the development and maintenance of other tissues/organs. Indeed, some of these patients show different levels of deafness, from conductive to sensorineural. We have used the mouse model, p63(-/-) (p63 knockout) and wt (wild type), for investigate the role of p63 in cochlea development to understand the sensorineural deafness phenotype in EEC patients.
Transcriptional analysis of TAp63a isoform shows that the different genes, involved in the cochlear neuroepithelium development, are regulated by this protein. We have demonstrated for the first time the specific TAp63 expression in the cochlear tissue of mice embryos. Furthermore, TAp63a is able to directly drive Hes5 expression, an important protein involved in the differentiation of the organ of Corti, both in vitro than in vivo. In addition, the morphological analysis of the cochlea in the p63(-/-) model shows Organ of Corti defects, similar to those identified in Hes5(-/-), both these mouse models leading to supernumerary hair cells. We have also excluded the apoptotic process TAp63-dependent in the development of the organ of Corti analysing the morphology of Puma and Noxa knockout mouse, both direct targets of TAp63 in this pathway. The organ of Corti of these mice were normal in structure and in number of Hair cells.
These data demonstrate that the transcription factor TAp63a is crucial for the development of the organ of Corti. In humans, the syndromes associated with p63 have been extensively studied over the years. The discovery that the sensorineural hearing loss is attributable to a loss of function for p63 results in the need to include this gene among those responsible for defects in hearing.

ABSTRACT N. 241

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MISSERO CATERINA**	
Telethon grant N.	**GGP12239**	
Total budget €	**259.700**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

DISSECTING THE MOLECULAR MECHANISMS UNDERLYING EPIDERMAL DEFECTS IN AEC SYNDROME

Missero Caterina (1), Ferone Giustina (1), Mollo Maria Rosaria (1), Thomason Helen (2), Dixon Jill (2), Zhou Huiqing (3), van Bokhoven Hans (3), Antonini Dario (1)

(1) CEINGE Biotecnologie Avanzate, via G. Salvatore 486, 80145 Napoli, Italy, Tel. +39.081.3737853, Fax +39.081.3737808, E-mail: missero@ceinge.unina.it
(2) Faculty of Medical and Human Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK
(3) Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, NL

AEC (Ankyloblepharon - Ectodermal Defects – Cleft Lip/Palate) syndrome is an autosomal dominant disorder characterized by cleft palate, ectodermal dysplasia, and severe skin erosions at birth, that are currently treated with generic wound care. AEC syndrome is caused by mutations in the p63 gene, encoding for a tetrameric transcription factor essential for epidermal development. Since little is known about the pathogenesis of AEC syndrome, we recently generated a unique knock-in mouse model (p63+/L514F) that carries a clinically relevant point mutation in the SAM domain of the p63 alpha isoform, and faithfully recapitulates the human disorder.
Using this mouse model we find that the AEC mutation exerts a selective dominant-negative function on wild-type p63 by affecting progenitor cell expansion during ectodermal development leading to a defective epidermal stem cell compartment (Ferone G. et al. EMBO Mol. Med., 4 (3) 192-205). These phenotypes are associated with impairment of fibroblast growth factor (FGF) signaling resulting from reduced expression of Fgfr2 and Fgfr3, direct p63 target genes. A defective stem cell compartment is observed in humans affected by AEC syndrome and in Fgfr2b-/- mice. Restoring Fgfr2b expression in p63+/L514F epithelial cells by treatment with FGF7 reactivates downstream mitogen-activated protein kinase signaling and cell proliferation.
In addition the observed skin fragility in the AEC mouse is associated with microscopic blistering between the basal and suprabasal compartments of the epidermis and reduced desmosomal contacts (Ferone G. et al. Hum. Mol. Gen., 2012 Oct 29). Expression of desmosomal cadherins and desmoplakin is strongly reduced in AEC mutant keratinocytes and in newborn epidermis. Importantly, a similar impairment in desmosome gene expression is observed in human keratinocytes isolated from AEC patients. We find that p63 controls these genes at the transcriptional level. Consistent with reduced desmosome function, AEC mutant keratinocytes have an impaired ability to withstand mechanical stress, which is alleviated by EGFR inhibitors known to stabilize desmosomes. Thus reduced mechanical strength resulting from p63 mutations can be alleviated pharmacologically by increasing desmosome adhesion with possible therapeutic implications.
Our study reveals that p63 is a crucial regulator of a FGF signaling and of cell adhesion molecules, and that this function is impaired in AEC syndrome.

ABSTRACT N. 242

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**MERLO GIORGIO ROBERTO**	
Telethon grant N.	**GGP11097**	
Total budget €	**489.800**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2011**

TRANSLATING CURRENT AND NEW KNOWLEDGE ON THE DEVELOPMENTAL FUNCTIONS OF P63 INTO RESTORING NORMAL DEVELOPMENT IN MODELS OF ORGAN CULTURE AND IN VIVO

Merlo Giorgio R. (1), Guerrini Luisa (2), Antonio Costanzo (3)

(1) Dept. of Molecular Biotechnology and Health Sciences, University of Torino, Via Nizza 52, 10126 Torino, Tel. +39.011.6706449, Fax. +39.011.6706432, E-mail: gmerlo@dti.telethon.it
(2) Dept. of Biosciences, University of Milano, Italy
(3) Dept. of Dermatology, University of Rome "Tor Vergata", Rome, Italy

This project aims to define the molecular regulations upstream and downstream of p63, a key molecule for ectoderm development, mutated in the congenital condition Ectodermal Dysplasia-Ectrodactyly-Cleft Palate (EEC) and related disorders. We are investigating on the functions of tissue-specific targets of p63 (Dlx5, Irf6, Pin1, Ikka) by functional genetics. As new knowledge on these pathways is generated, we aim to identify druggable targets for early correction of limb/palate/skin dysmorphysm, or suitable for gene delivery approaches. Thus this project sets the framework for a correction of the dysmorphologies that characterize EEC and related disorders.
Upstream of p63. The stability of p63 is controlled by several post-transcriptional and post-translational mechanisms, including microRNAs, sumoylation, phosphorylation, ubiquitination and, recently identified, peptidyl-prolyl isomerization. Indeed the isomerase Pin1 induces degradation of p63, in vitro and in vivo, and interestingly, Pin1 is under control of the Dlx5/Dlx6 genes, which cause Ectrodactyly in mouse and man. p63 stability is also controlled by Irf6, which in turn is a downstream p63 target and constitutes a regulatory loop essential for skin morphogenesis and keratinocyte differentiation.
Downstream of p63. We have identified Irf6, Ikka and Dlx5/Dlx6, and further identified Wnt5a downstream of Dlx5-Dlx6. We are now functionally characterizing these genes and verifying their ability to correct limb and palatal defects, in vivo. To do this, we are using the mouse models of the EEC (p63R279H), SHFM (Dlx5;Dlx6) and Van der Woude (Irf6R84C) disorders, and generate transgenic mouse lines re-expressing the downregulated targets. Upon breeding of these lines, we will obtain information on the potential therapeutic relevance of identified pathways.
Being located at the surface of the embryo, the ectoderm is easily accessible to transducing viral vectors as well as to diffusible molecules or pharmacological compounds. We are setting up cultures of embryonic limb, palate and skin, from normal and mutated embryos to be used to test therapeutic strategies using the p63 targets and the FGF/BMP signaling molecules as paradigms for interventions on the diseased tissue. To test gene-delivery approach, limb and palate cultures will be transduced with Pin1 and Ikka (limbs) and Irf6 (palate and skin) via viral vectors. In a second paradigm, to test diffusible molecules, FGFs and TGF-b/BMPs, found to be downstream of Irf6/Ikk-a, are being applied on cultured embryonic tissues, a proof-of-principle for the future screening of compounds for effective biochemical interventions.
Overall, by combining present and newly generated knowledge, we hope to provide experimental evidence that restoring the function of tissue-specific p63 targets may restore normal development.

ABSTRACT N. 243

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TARTAGLIA MARCO**	
Telethon grant N.	**GGP10020**	
Total budget €	**231.700**	
Centres: **1**	*Duration (yrs):* **2**	*Starting year:* **2010**

MOLECULAR BASES OF NOONAN SYNDROME AND RELATED DISORDERS

Martinelli Simone (1), Flex Elisabetta (1), Fodale Valentina (1), Pantaleoni Francesca (1), Stellacci Emilia (1), Carta Claudio (1), Motta M. Letizia (1), Cianetti Luciano (1), Cordeddu Viviana (1), Caputo Viviana (1), Ciolfi Andrea (1), Niceta Marcello (1), Zampino Giuseppe (2), Digilio Maria C (3), Selicorni Angelo (4), Mazzanti Laura (5), Rossi Cesare (6), Lepri Francesca (3), De Luca Alessandro (7), Ferrero Giovanni B (8), Nardozza Aurelio P (9), Castagnoli Luisa (9), Stella Lorenzo (9), Kutsche Kerstin (10), Lemischka Ihor (11), Cavé Helene (12), Ahmadian M Reza (13), Zenker Martin (14), Dallapiccola Bruno (3), Gelb Bruce D (11), Tartaglia Marco (1)

(1) Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy, Tel. 06.49902569, Fax 06.49902850, E-mail: mtartaglia@iss.it
(2) Università Cattolica del Sacro Cuore, Roma, Italy
(3) Ospedale Bambino Gesù, Roma, Italy
(4) Università di Milano Bicocca, Monza, Italy
(5) Policlinico S. Orsola-Malpighi, Bologna, Italy
(6) Università di Bologna, Bologna, Italy
(7) Istituto Mendel-IRCCS "Casa Sollievo della Sofferenza", Roma, Italy
(8) Università di Torino, Torino, Italy
(9) Università "Tor Vergata", Roma, Italy
(10) University Medical Center Hamburg-Eppendorf, Hamburg, Germany
(11) Mount Sinai School of Medicine, New York, NY, USA(12) Hopital Robert Debre', Paris, France
(13) Heinrich-Heine University Medical Center, Düsseldorf, Germany
(14) Institute of Human Genetics, University Hospital, Magdeburg, Germany

Noonan syndrome (NS) is among the most common non-chromosomal disorders affecting development and growth. NS is caused by aberrant RAS signaling and is genetically heterogeneous. Our past work established PTPN11, SOS1, KRAS, NRAS, BRAF, RAF1 and SHOC2 as NS disease genes, with mutations accounting for 75% of cases.
Goals of this project were to identify the missing genes underlying NS, characterize the molecular mechanisms driving aberrant function of the mutated proteins, define more precisely the clinical impact of mutations and identify relevant genotype-phenotype correlations.
Major products of our work follow.
By using a candidacy approach focused on genes coding transducers with role relevant to RAS signaling, we discovered that germline mutations in CBL, a tumor-suppressor gene encoding for an adaptor protein with E3 ubiquitin ligase activity, underlies a previously unrecognized RASopathy. Mutations were found to impair CBL ligase activity and dysregulate signaling through RAS (Martinelli et al. 2010, Am J Hum Genet, 87:250-7). By using the same approach, a novel disease gene implicated in a RASopathy with predisposition to haematological malignancies has been identified (manuscript in preparation).
We provided a more accurate picture of the spectrum of SOS1, RAF1and SHOC2 mutations and their associated phenotypic features (Lepri et al. 2011, Hum Mutat 32:760-72; two manuscripts in preparation).
We functionally characterized a panel of NS-causing PTPN11 mutations. Specifically, we demonstrated that amino acid substitutions at codons 62 and 63 have a profound and complex effect on protein structure, and that a selection-by-function mechanism acting on mutations at those codons implies balancing of counteracting effects operating on SHP2's activity (Martinelli et al. 2012, J Biol Chem 287:27066-77).
We identified and characterized novel mechanisms implicated in HRAS functional dysregulation underlying Costello syndrome and NS (Gremer et al. 2010, Hum Mol Genet 19:790-802 and manuscript in preparation).
Our work has also been directed to determine the mechanisms controlling SHOC2 function. In particular, we characterized the domains mediating SHOC2 nuclear-cytoplasmic shuttling and those implicated in plasma membrane targeting of the disease-causing myristoylated protein (manuscript in preparation).
Finally, we contributed to the establishment of iPS cell lines for LEOPARD syndrome (Carvajal-Vergara et al. 2010, Nature 465:808-12), NS (Mulero-Navarro et al. 2012, Nature, under revision) and other RASopathies (manuscript in preparation).
Overall, our findings have defined novel mechanisms by which upregulation of signal traffic through RAS affect development and growth, and have allowed the establishment of molecular tools for proper diagnosis, patient management, and therapeutic target discovery and small molecule testing.

ABSTRACT N. 244

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**CESARENI GIANNI**	
Telethon grant N.	**GGP09243**	
Total budget €	**254.600**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

SHP-2 DIMERIZATION: AN ADDITIONAL REGULATORY MECHANISM FOR MODULATING SHP-2 ACTIVITY

Nardozza Aurelio Pio (1), D'Orazio Melania (1), Santonico Elena (1), Trapannone Riccardo (1), Corallino Salvatore (1), Filomeni Giuseppe (1,2), Tartaglia Marco (2), Battistoni Andrea (1), Castagnoli Luisa (1), Cesareni Gianni (1,3)

(1) University of Rome Tor Vergata, Via della ricerca scientifica, 00133 Rome, Italy, Tel. +39.06.72594315, Fax. +39.06.72594599, E-mail. Cesareni@uniroma2.it
(2) Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
(3) IRCCS Fondazione Santa Lucia, Rome, Italy

The tyrosine specific SHP-2 phosphatase plays key regulatory roles in the modulation of the cell response to growth factors and cytokines. Over the past decade, the integration of genetic, biochemical and structural information has helped interpreting the pathological consequences of altered SHP-2 function. By using complementary approaches, here we provide evidence that endogenous SHP-2 can dimerize through the formation of disulfide bonds that may also involve the catalytic cysteine. We show that the fraction of dimeric SHP-2 is modulated by growth factor stimulation and by the cell redox state. Measurement of the phosphatase activity in the monomeric or dimeric forms indicates that the latter is threefold less active, thus pointing to the dimerization process as an additional possible mechanism for controlling SHP-2 activity. Remarkably, dimers formed by two SHP-2 mutants, SHP-2E76K and SHP-2T468M, which underlie distinct human diseases and display diverse biochemical properties, were found to respond differently to EGF stimulation. While the hyperactive leukemia-associated SHP-2E76K reacts to EGF by modulating its dimer/monomer ratio with a kinetic comparable to the wild type protein, an increase in dimer formation was observed for the catalytically impaired LEOPARD syndrome-causing SHP-2T468M mutant. Although this differential behavior cannot be rationalized mechanistically yet, these findings suggest a possible regulatory role of dimerization on SHP-2 function.

ABSTRACT N. 245

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**TALORA CLAUDIO**	
Telethon grant N.	**GGP12264**	
Total budget €	**252.600**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2012**

CALCIUM DYSREGULATION AND OXIDATIVE STRESS: FROM MOLECULAR MECHANISMS TO THERAPEUTIC IMPLICATIONS IN HAILEY-HAILEY DISEASE

Biolcati Gianfranco (1), Aurizi Aurizi (1), Barbieri Barbieri (1), Palleschi Claudio (2), Zanni Elena (2), Uccelletti Daniela (2), Cialfi Samantha (3), Manca Sonia (3), Screpanti Isabella (3), Talora Claudio (3)

Porphyria Center San Gallicano Institute IRCCS, Rome, Italy
Dept. Biology and Biotechnology "C. Darwin"; Sapienza University of Rome
(3) Dept. of Molecular Medicine, Sapienza University of Rome, Viale Regina Elena 291, 00161, Rome, Italy, Tel. +39.06.49255674, Fax +39.06.4464129, E-mail: claudio.talora@uniroma1.it

Hailey-Hailey Disease is a chronic, recurrent blistering rare disorder

characterized clinically by erosions occurring primarily in intertriginous regions and histologically by suprabasal acantholysis. We previously reported that oxidative stress plays a specific role in the pathogenesis of Hailey-Hailey disease by regulating the expression of factors playing an important role in keratinocyte proliferation and differentiation. alpha-Melanocyte-Stimulating hormone (a-MSH) is a melanocortin peptide that in addition to its role in pigmentation also reduces oxidative stress independent of melanin synthesis. Given the significance of oxidative-stress in Hailey-Hailey disease, here we investigated the potential effects of a-MSH-analogous (Nle4-D-Phe7-a-MSH), antioxidant properties in HHD-lesion derived keratinocytes. We show that treatment of HHD-keratinocytes with Nle4-D-Phe7-a-MSH, contributes to the upregulation of Nrf2, a redox sensitive transcription factor that plays a pivotal role in redox homeostasis during oxidative stress. Additionally, Nle4-D-Phe7-a-MSH treatment restores the defective proliferative capability of lesion-derived keratinocytes. Our results show that Nrf2 is an important target of the Nle4-D-Phe7-a-MSH signaling that reduces oxidative stress. As Nle4-D-Phe7-a-MSH possesses anti-oxidant effects, we assessed the clinical potential of this a-MSH analogue, in patients with Hailey-Hailey disease. Nle4-D-Phe7-a-MSH (16 mg) was given in a phase II open-label pilot study subcutaneously as a sustained- release resorbable implant formulation to 2 patients presenting several long-standing skin lesions. Both patients treated with Nle4-D-Phe7-a-MSH had 100% clearance of HHD lesions 60 days after the first injection, independently of the lesion localization. Life quality as measured by Dermatology Life Quality Index likewise improved in all 2 patients 30 days after the first injection of Nle4-D-Phe7-a-MSH. In conclusion, Nle4-D-Phe7-a-MSH) is effective and safe for the treatment of Hailey-Hailey skin lesions.

ABSTRACT N. 246

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**URSINI MATILDE VALERIA**	
Telethon grant N.	**GGP08125**	
Total budget €	**351.800**	
Centres: **3**	*Duration (yrs):* **3**	*Starting year:* **2008**

UNRAVELLING THE MOLECULAR MECHANISMS OF IMPAIRED NEMO FUNCTION IN IP PATHOGENESIS

Pescatore Alessandra (1), Fusco Francesca (1), Leonardi Antonio (2), Vito Pasquale (3,4), Ursini Matilde Valeria (1)

(1) Human Molecular Genetics Laboratory, Institute of Genetics and Biophysics "A. Buzzati-Traverso" CNR, Via P. Castellino 111, 80125 Naples, Italy Tel.+39.081.6132262, Email: ursini@igb.cnr.it
(2) Dipartimento di Biologia e Patologia Cellulare e Molecolare, "Federico II," University of Naples, Naples, Italy
(3) Dipartimento di Scienze Biologiche ed Ambientali, Università degli Studi del Sannio, Benevento, Italy
(4) Biogem Consortium, Ariano Irpino, Italy

Incontinentia pigmenti (IP, OMIM #308300) is an X-linked dominant and male-lethal neuroectodermal disease with an heterogeneous and often severe clinical presentation. IP is caused by mutations in the NEMO gene. NEMO maps in a region with a unique genomic architecture presenting a high frequency of micro/macro-homologies, tandem repeats, and repeat/repetitive sequences which might predispose to pathologic genomic recombination. We summarize here the latest results of our Telethon project that further improved our ability to perform a correct diagnosis of IP, which is an indispensable pre-requisite for the application of therapies and for prevention. We demonstrated that both recurrent deletion (exon4-10 del, present in 80% of IP patients) and non-recurrent deletion might occur at the IP locus trough different mechanisms such as NAHR, NHEJ, FoSTeS, MMBIR and intra-locus gene conversion. These events, occurring during both meiosis and mitosis, might involve not only NEMO but also the overlapping G6PD, without any visible clinical signs due to G6PD deficiency.
Then, we produced two important discoveries on the affected NEMO functions in the cells of IP patients. Indeed, beside the mutations causing loss-of-function of NEMO, few missense mutations were found in IP, which we studied in further detail.
NEMO encodes the regulatory subunit of the IKK complex responsible of the activation of NF-kappaB transcription factor in multiple signaling pathways controlling immunity, cell survival, differentiation and proliferation.
Inflammatory cytokines (TNF-alfa, IL1 etc.) probably function through conformational change in the kinase complex resulting from either ubiquitination of NEMO or NEMO binding to ubiquitin. One of the E3 ligase responsible for non-degradative polyubiquitination of NEMO is TRAF6, which participates in several signalling pathways controlling immunity, osteoclastogenesis, skin development, and brain functions. We studied the IP-associated E57K NEMO mutant found in a mild form of IP, showing an impaired IL-1 signaling, and we established that a fragment encompassing 57-69aa of NEMO is responsible for its binding to the CC domain of TRAF6, required for NF-kappaB activation. This site appears to work in concert with NUB domain, that binds to the polyubiquitinated chains of TRAF6 (as we previously established), suggesting a dual mode of TRAF6 recognition.
Upon TNF stimulation, NEMO and so the IKK complex are recruited to the TNF receptor and activate NF-kappaB that exerts cytoprotective functions by inducing transcription of anti-apoptotic genes. In the absence of NEMO, cells are sensitized to TNF-induced death, which is a key step in the triggering of the IP disease. Through the analysis of IP-associated mutants that expectedly, prevented TNF-induced NF-kappaB activation but, unexpectedly, retained the cell-death-preventive activity of NEMO, we have had the possibility to study the cell-death-preventative role of NEMO separately from its gene-activator role.

ABSTRACT N. 247

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**BARCHI MARCO**	
Telethon grant N.	**GGP12189**	
Total budget €	**324.200**	
Centres: **2**	*Duration (yrs):* **3**	*Starting year:* **2012**

UNDERSTANDING XY CHROMOSOME SEGREGATION DEFECTS IN MAMMALS: NEW INSIGHTS FROM THE REGULATION OF EXPRESSION AND FUNCTION OF SPO11 SPLICE ISOFORMS

Sette Claudio, Barchi Marco

University of Rome "Tor Vergata", Department of Biomedicine and Prevention, section of Anatomy, Via Montpellier n.1, 00133, Roma, Italy, Tel.06.72596266, Fax 06.72596268, E-mail: marco.barchi@uniroma2.it

During meiosis, recombination between homologous chromosomes is essential to ensure their proper segregation in the gametes. A key component of this machinery is SPO11, the endonuclease that introduces the double strand breaks, thereby initiating meiotic recombination. Indeed if Spo11 gene is ablated, both male and female mice become sterile (Baudat F. et al., Mol Cell (6) 5, 2000). In mammals Spo11 gene codify for at least two splicing isoforms: Spo11-beta and Spo11-alpha (Keeney S. et al., Genomics (61) 2, 1999). We have recently generated a transgenic mouse expressing SPO11-beta cDNA under the control of the meiotic-specific promoter Xmr [XmrTg(Spo11-beta)], and found that it rescues most of the defects observed in Spo11-/- female mice. However, in males expression of the XmrTg(Spo11-beta) transgene in absence of the endogenous gene (SPO11-beta only) did not support proper XY recombination and disjunction. As a consequence, the majority of spermatocytes were eliminated by a spindle checkpoint at metaphase and male mice were mostly infertile (Kauppi L. et al., Sience (331) 6019, 2011). These observations point to a non-redundant function of SPO11-alpha in meiosis, and support its potential involvement in the regulation of XY chromosome segregation. Since the absence of recombination at the XY pairing region of SPO11-beta only mice is mechanistically linked to what observed in patients affected by Klinefelter syndrome (XXY karyotype), our findings also suggest that alteration in the expression or function of SPO11 isoforms could be implicated in the pathogenesis of Klinefelter and other male derived aneuploidies (such as XYY, or XO) caused by non-disjunction defects in meiosis.
In this study, we aim at investigating the possible contribution of SPO11 to the pathogenesis of the sex chromosomes non-disjunction syndromes of paternal origin. We will use genetically modified mice to study the function of the SPO11 splice variants and the mechanisms that control Spo11 splicing during meiosis. In addition, we will look for as yet unidentified SPO11-functional interactors in mammals. The project is subdivided in four Specific Aims:
Aim 1 (Coordinator): Generation of a SPO11-alpha only transgenic mouse. We will generate a construct to express SPO11-alpha under the Xmr promoter. Founders will be crossed with Spo11+/- mice to generate Spo11-/- / SPO11-alpha mice. The phenotype of these SPO11-alpha only mice will be analyzed in detail for meiotic progression and homologous recombination defects.
Aim2 (Coordinator): Analyses of XY chromosome segregation in spermatozoa from SPO11-alpha only and SPO11-beta only mice.

These analyses will determine whether mice expressing only one SPO11 isoform produce spermatozoa with aberrant segregation of the sex chromosomes.
Aim 3 (Coordinator/Partner 1). Identification of SPO11-alpha and SPO11-beta functional partners. Functional partners of SPO11 in mammals are unknown. Their identification might shield a new light in the understanding of meiotic chromosome segregation in mammals.
Aim 4 (Partner 1): Identification of the factors that modulate Spo11 splicing in mouse testis. Using a Spo11 minigene, we will identify the splicing factors that regulate Spo11 splicing. Using mouse models defective for XY chromosome segregation or for the expression of splicing factors, we will test whether Spo11 splicing is altered and/or if the defect correlates with altered expression of specific splicing factors.

ABSTRACT N. 248

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator		**RICCIO ANDREA**
Telethon grant N.		**GGP11122**
Total budget €		**231.000**
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

GROWTH DISORDERS AND GENOMIC IMPRINTING: GENETIC DEFECTS AND MOLECULAR MECHANISMS

Sparago Angela (1), Cerrato Flavia (2), De Crescenzo Agostina (2), Citro Valentina (1), Anvar Zahra (1,2), Riso Vincenzo (1,2), Freschi Andrea (2), Kukreja Harpreet (1), Grimaldi Giovanna (1), Riccio Andrea (1,2)

(1) Istituto di Genetica e Biofisica A.Buzzati-Traverso, CNR, via P. Castellino 111, Napoli, Italy, Tel. 081.6132444, Fax 081.6132706, E-mail: andrea.riccio@unina2.it
(2) Dipartimento di Scienze Ambientali, Seconda Università di Napoli

The objective of this project is to define the molecular genetics and pathogenesis of congenital growth disorders associated with genomic imprinting, such as Beckwith-Wiedemann Syndrome (BWS) and Silver-Russell Syndrome (SRS). A large proportion of BWS and SRS patients have imprinting defects at chromosome 11p15.5. These can either result from inherited mutations of Imprinting Control Regions (ICRs) and have high recurrence risk or arise independently from the sequence context and generally not transmitted to the progeny. It was previously demonstrated that maternally inherited 1.4-2.2 kb deletions inside the telomeric ICR (IC1) of the 11p15.5 imprinted gene cluster are associated with hypermethylation of the ICR sequence and BWS with different penetrance and expressivity. We have now explored the relationship between IC1 microdeletions and phenotype by analysing a number of previously described and novel mutant alleles. We demonstrate that the microdeletions mostly affect IC1 function by changing the spacing of the target sites for the zinc-finger-protein CTCF (CTS). The extent of IC1 inactivation and the clinical phenotype are influenced by the arrangement of the residual CTSs. Careful characterization of the IC1 microdeletions is therefore needed to predict recurrence risks and phenotypical outcomes. More recently, we identified a case with a paternally transmitted 60 kb deletion at 11p15.5 showing recurrent severe Intra-Uterine Growth Restriction (IUGR). We found that this deletion includes the centromeric ICR (IC2) and leads to an imprinting alteration resulting in silencing of the KCNQ1OT1 gene and activation of CDKN1C and PHLDA2. This is the first report implicating impinting defects of the centromeric imprinted 11p15.5 domain in IUGR. Concerning the cases with imprinting defects and no sequence alteration in cis, we are now focusing on the identification of protein factors that are needed for the maintainance of DNA methylation at the ICRs in early embryogenesis. ZFP57 has been demonstrated to be required for imprinting maintenance at several imprinted loci in the mouse. No mutation in such gene has been found in BWS and SRS so far. The possible involvement of other loci is being investigated by exome-seq analysis of patient DNAs.

ABSTRACT N. 249

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator		**BRANZEI DANA**
Telethon grant N.		**GGP12160**
Total budget €		**223.500**
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

LINKS BETWEEN DNA REPAIR DEFECTS AND CHROMOSOME STRUCTURAL ANOMALIES IN COHESINOPATHY CELLS

Abe Takuya, Branzei Dana

Fondazione IFOM, Istituto FIRC di Oncologia Molecolare, Via Adamello 16, 2-139, Milano, Italy, Tel. 02.574303259, Fax 02.574303231, E-mail: dana.branzei@ifom.eu

Cohesin belongs to the highly conserved structural maintenance of chromosomes (SMC) family of ATPases and acts to physically connect sister chromatids from S phase till anaphase. Cohesin complex comprises four core proteins: Smc1, Smc3, Scc1 (also known as Mcd1 or as Rad21 in budding yeast and mammalian cells) and Scc3, and plays a structural role. The cohesin complex is loaded onto chromatin in G1 and S phases with the help of Scc2 and Scc4, also called cohesin loaders.
The term “cohesinopathies” refers to the effect of mutations in protein components of the cohesion pathway on the human condition. Three cohesinopathies have been so far characterized: Cornelia de Lange Syndrome (CdLS), Roberts syndrome/SC phocomelia (RBS), and Warsaw Breakage syndrome (WABS). The cohesinopathies manifest a wide spectrum of phenotypes that include mental deficiency, growth retardation, and various developmental defects. The molecular mechanisms underlying these defects and phenotypic similarities of the diseases are presently unclear.
Cells derived from cohesinopathy patients show a wide range of phenotypes, the main feature being DNA damage sensitivity of varying severity in addition to cohesion defects. By constructing DT40 cell lines with mutations mimicking the ones found in cohesinopathy patients we plan to establish cellular models for cohesinopathies. We are testing if there is a link between the repair defects and chromosome structural anomalies of these cells, which may also explain the phenotypic variability of these diseases.

ABSTRACT N. 250

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator		**BIFFO STEFANO**
Telethon grant N.		**GGP10012**
Total budget €		**263.400**
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

MODULATION OF EUKARYOTIC INITIATION FACTOR 6 ACTIVITY AS A THERAPEUTIC TOOL IN RIBOSOME-BASED DISEASE

Biffo Stefano (1,2), Pesce Elisa (1), Calamita Piera (1), Johanna Rommens (3), Giovanna D’amico (4)

(1) San Raffaele Scientific Institute, Via Olgettina 58 20132 Milano, Italy E-mail: biffo.stefano@hsr.it
(2) Disit, Alessandria, Italy
(3) Centro Matilde Tettamanti, Monza, Italy
(4) Hospital For Sick Children, Toronto, Canada

eIF6 binds 60S ribosomal subunits impairing their improper joining to 40S. Point mutations of eIF6 rescue the loss of SBDS, the gene mutated in Swachman-Diamond syndrome. In addition, both eIF6 overexpression and SBDS deficiency lead to increased tumor formation. Our hypotheses are that modulating eIF6 may lead to a therapeutic outcome in Swachman-Diamond syndrome; tumor cells with mutated SBDS may be sensitive to selective chemotherapeutic drugs.
We have developed an HTS-ready system for detecting eIF6 modulators of binding. In this system we have tested the binding efficiency of eIF6 point mutants, and of the simultaneous administration of the SBDS protein. Data demonstrate that the HTS system is highly reproducible with a Z-factor of 0.90. The efficiency of binding of mutant eIF6 to 60S is comparable to the one of wt eIF6.
We also have started to produce SBDS tumor cells and matched controls to define their resistance to drugs. We first attempted, in collaboration with G. D’Amico (Laboratorio di Immunologia e Terapia Cellulare, Fondazione Tettamanti, Monza, Italy) to transform human SBDS deficient cells. We were unable to derive tumor cells. Currently, we are trying to transform mouse embryonic fibroblasts derived from a mouse SBDS model (in collaboration with Johanna Rommens, The Hospital for Sick children, Toronto, Canada).
Next, we plan to anayze the effects of SBDS in eIF6 release, in vitro, and to characterize the intrinsic properties of tumor cells with mutated SBDS.

ABSTRACT N. 251

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**PICHIERRI PIETRO**	
Telethon grant N.	**GGP12144**	
Total budget €	**225.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2012**

UNCOVERING THE MOLECULAR PATHOLOGY OF WERNER SYNDROME: ANALYSIS OF THE FUNCTIONAL RELATIONSHIP BETWEEN ATR-RELATED WRN FUNCTION, REPLICATION STRESS AND PREMATURE CELLULAR SENESCENCE

Iannascoli Chiara (1,3), Palermo Valentina (1,3), Zolla Valerio (1,3), Basile Giorgia (2,3), Franchitto Annapaola (2,3), Pichierri Pietro (1,3)

(1) Istituto Superiore di Sanità, Sezione di Cancerogenesi Sperimentale e Computazionale, Viale Regina Elena 229, 00161 Roma, Tel. 06.49902994/2954, Fax 06.60513138, E-mail: pietro.pichierri@iss.it
(2) Istituto Superiore di Sanità, Sezione di Epidemiologia Molecolare, Roma
(3) Istituto Superiore di Sanità, Gruppo di Studio sulla Stabilità del Genoma, Roma

Werner syndrome (WS) is one of the genetic diseases characterised by chromosome fragility, cancer proneness and premature ageing. The molecular bases of WS are poorly understood and their uncover is complicated by the apparent pleiotropy of WRN, the product of the gene mutated in WS. In particular, it is unclear the molecular mechanism leading to premature replicative senescence, a cellular phenotype that may be correlated with ageing in patients. In normal cells, senescence is associated with telomere damage. However, WS cells senesce with long telomeres and often show signs of stress-induced senescence. In cells, one of the source of stress is related with unprotected DNA replication, which may result in DNA damage. Many evidence mostly from our studies indicate that WRN participates in the ATR-dependent response to stalled or collapsed replication forks and studies from other groups evidenced that hypomorphic ATR alleles associate with premature aging in a mouse model. Our objective is to undertake a mechanistic analysis of replication-related WRN regulatory networks and determine whether the premature cellular senescence of WS derives from loss of ATR-related functions of WRN at perturbed forks, as preliminary results from our group suggest. To test our hypothesis, beside the use of acknowledged cellular models of WS and that of common fragile sites as readout of spontaneous replication stress, we will take advantage from regulated WRN RNAi in primary human fibroblasts complemented with RNAi-resistant ATR-unphosphorylable form of WRN. This unique allele-switch WS model issued in primary fibroblasts recapitulates key WS phenotypes and will allow us to investigate the cellular and molecular events leading to premature senescence in WS, comparing them with those observed in normal cells. In particular, we expect to correlate accumulation of DNA damage at naturally-occurring replication stalling sites with premature replicative senescence and the secretory phenotype (SASP) of WS. Finally, using our inducible RNAi model, we expect to be able to determine if the telomeric function of WRN is under the control of the ATR pathway. A better understanding of how and why WS cells undergo premature replicative senescence is expected to shed light also to the mechanisms underlying premature ageing in patients.

ABSTRACT N. 252

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GALLI ALVARO**	
Telethon grant N.	**GGP09166**	
Total budget €	**181.500**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

THE YEAST SACCHAROMYCES CEREVISIAE AS CELLULAR ENVIRONMENT TO STUDY ADENO-ASSOCIATED VIRUS REPLICATION, INTEGRATION AND ENCAPSIDATION

Cervelli Tiziana, Backovic Ana, Della Latta Veronica, Bologna Caterina, Cipriani Filippo, Galli Alvaro

Istituto di Fisiologia Clinica, CNR via Moruzzi 1, Pisa, Tel. 050.3153094, Fax 050.3153327, E-mail: alvaro.galli@ifc.cnr.it

The ideal approach of gene therapy is to replace the defective gene with a functional copy. The most promising vectors for gene therapy studies are those based on the adeno-associated virus (AAV), a non pathogenic single stranded DNA (ssDNA) virus. To date, the most extensive clinical application of AAV is for cystic fibrosis. We believe that a more profound knowledge of the AAV biology may give a great contribution for AAV therapeutic application. Two main limitations that block the usage of AAV vectors for gene therapy are: the high variability of transduction efficiency depending on cell type and the lack of a safe and efficient production and purification methods. The most used methods to produce AAV vectors are time consuming and very expensive. Therefore, to find a more simple eukaryotic system where AAV replicates and encapsidates may provide an excellent opportunity for studying AAV biology and, more importantly, for finding other host organism where AAV vectors could be produced. Recently, the yeast Saccharomyces cerevisiae has been shown to be a very useful genetic tool for virus research. Our final goal is to use the yeast Saccharomyces cerevisiae as cell recipient where AAV may be assembled. During the last two years we have demonstrated that recombinant AAV (rAAV) replicates in yeast in presence of Rep68 and that the capsid proteins, VP1, VP2 and VP3 are expressed and assembled in yeast. Anyways, to produce high titer of rAAV we need to improve either DNA replication or capsid assembly. Moreover, to implement the amount of ssDNA we tested two yeast strains carrying mutation in protein rad52 and rad50 involved in ssDNA processing during DNA repair. No significant effect on amount ssDNA was seen. To assemble rAAV in human cells, it is necessary to express AAV Rep and Cap proteins and helper virus proteins such as E4orf6 and E1b55k of adenovirus. Therefore, we have also investigated the effect of expression of these adenoviral proteins on AAV genome replication in yeast. We observed an increased level of the AAV genome as single stranded DNA in presence of these proteins. Moreover, we demonstrated that E4orf6-E1b55k complex alters the expression of yeast DNA double strand break repair protein MRE11 as in human cells. This observation confirms that yeast is a good model system to study rAAV replication. Another strategy to increase the production of AAV ssDNA has been the construction of a rAAV containing yeast replication origin into the backbone. In this way we could maintain high level of plasmid from which the AAV DNA is replicated. Interestingly, we observed the formation of AAV ssDNA also when Rep68 is net expressed suggesting that this protein is not necessary for AAV replication when high copy of the double stranded AAV DNA is present. We tried to improve the Cap proteins expression constructing new vectors. We obtained the right ratio VP1:VP3 expressing VP3 under control of p40 promoter and VP1 under control of Gal1 inducible promoter and adjusting the induction time. Using this vectors combination we obtained a low level of the assembled viral capsid. So we constructed other vectors to improve the expression of Cap proteins. The highest level of proteins was obtained cloning VP1 and VP2, 3 under control of Gal10 and Gal1 promoter, respectively. We also demonstrated that the regulatory assembling protein, AAP is expressed in yeast. By using these novel vectors, we were able to purify empty AAV capsids from yeast. We are currently approaching to the encapsidation of AAV DNA and to the purification of rAAV from yeast cells. Altogether, our results strongly indicate that yeast has the potentiality to be used to assemble rAAV for gene therapy.

ABSTRACT N. 253

Telethon Research Projects - Other Genetic Diseases		
Principal Investigator	**GIACCA MAURO**	
Telethon grant N.	**GGP11068**	
Total budget €	**204.600**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2011**

TOWARDS BETTER AAV GENE THERAPY: WHOLE GENOME siRNA AND microRNA HIGH THROUGHPUT SCREENING FOR THE IDENTIFICATION OF THE MOLECULAR DETERMINANTS GOVERNING AAV VECTOR TRANSDUCTION, VECTOR PRODUCTION AND VECTOR-INDUCED GENE CORRECTION

Giacca Mauro, Lorena Zentilin, Mano Miguel, Zacchigna Serena, Lovric Jasmina, Ippodrino Rudy, Backovic Ana

International Center for Genetic Engineering and Biotechnology (ICGEB), Padriciano 99, 34149 Trieste, Italia, Tel. +39.040.3757324, Fax +39.040.226555, E-mail: giacca@icgeb.org

Adeno-associated viral vectors (AAVs) have emerged as a potent gene therapy vehicle, currently valued for human clinical applications in the treatment of inherited, degenerative or acquired diseases, and as a precious basic research tool for gene delivery in animal models. However, despite the patent efficacy of rAAV vectors in transducing post-mitotic muscular and neuronal cells, insufficient

transduction of other tissues and cell types still preclude wider therapeutic applications. Limited information is available on the molecular determinants of AAV tissue permissivity.
We have previously demonstrated that the host cell DNA damage response machinery and, in particular, the proteins involved in double-stranded DNA break repair (DSB) (Mre11-Rad50-Nbs1 (MRN) complex), interact and negatively regulate incoming rAAV genome processing. We have now shown that permissivity to AAV transduction strictly correlates with cell terminal differentiation, coinciding with the reduced expression of the MRN complex. Indeed, siRNA-mediated downregulation of MRN proteins, or treatment with miR-24, which negatively regulates the Nbs1 levels, induce AAV permissivity in both cultured cells and in the juvenile liver in vivo (Lovric et al., 2012, Mol Ther: 20, 2087–2097).
To systematically identify the host cell factors involved in internalization, intracellular trafficking, processing of AAV genome and, eventually, AAV gene expression, either positively or negatively, we have performed a high-throughput screening using a genome-wide siRNA library (18175 human gene targets) in AAV2-Luciferase transduced HeLa cells. Analysis of the results obtained from a primary screening identified 1528 genes affecting transduction by AAV vectors more than 4-fold (184 genes more than 8-fold). Of these genes, 993 are inhibitors of AAV transduction, whereas 535 are required for efficient transduction. The effect of these genes on AAV transduction was further confirmed in a secondary screening. Gene ontology analysis of the inhibitory siRNAs revealed a clear overrepresentation of genes related to DNA recombination and repair, including members of the MRN complex, and cell cycle control, whereas in the subset of genes required for infection an overrepresentation of genes involved in endocytosis, intracellular trafficking and transcription as well as genes involved in the ubiquitin-proteasome system was observed. In addition, a significant number of genes were identified, which have not previously been associated with AAV transduction.
A better understanding of the mechanisms of action of the identified genes will open new perspectives for the exploitation of RNAi strategies to improve in vivo transduction and the development of recombinant AAV as efficient gene delivery systems.

TELETHON RESEARCH SERVICES/CORE FACILITIES

ABSTRACT N. 254

Telethon Research Services/Core Facilities		
Principal Investigator	**FILOCAMO MIRELLA**	
Telethon grant N.	**GTB07001**	
Total budget €	**2.062.768**	
Centres: **10**	*Duration (yrs):* **5**	*Starting year:* **2007**

TELETHON NETWORK OF GENETIC BIOBANKS (TNGB) WWW.BIOBANKNETWORK.ORG

Filocamo Mirella (1), Baldo Chiara (2), Goldwurm Stefano (3), Renieri Alessandra (4), Angelini Corrado (5), Moggio Maurizio (6), Mora Marina (7), Merla Giuseppe (8), Politano Luisa (9), Garavaglia Barbara (10)

(1) UOSD Centro di Diagnostica Genetica e Biochimica delle Malattie Metaboliche, Istituto G. Gaslini, Largo G. Gaslini 5, 16147 Genova, Tel. 010.5636792/609, Fax 010.383983, E-mail: info@biobanknetwork.org
(2) SC Laboratorio di Genetica Umana, E.O. Ospedali Galliera, Genova
(3) Centro Parkinson, Istituti Clinici di Perfezionamento, Milano
(4) UOC Genetica Medica, Dip. di Biotecnologie, Università di Siena
(5) Dip. di Neuroscienze, Università di Padova, IRCCS San Camillo, Venezia
(6) UOD Diagnostica Malattie Neuromuscolari, Fond. IRCCS Ca' Granda Osp. Maggiore Policlinico, Milano
(7) Laboratorio di Biologia Cellulare, UO Malattie Neuromuscolari e Neuroimmunologia, Fond. IRCCS Istituto Neurologico C. Besta, Milano
(8) Unità di Genetica Medica, IRCCS Casa Sollievo della Sofferenza, S. Giovanni Rotondo (FG)
(9) Cardiomiologia e Genetica Medica, Dip. di Medicina Sperimentale, Seconda Università di Napoli
(10) UO Neurogenetica Molecolare, Fond. IRCCS Istituto Neurologico C. Besta, Milano

TNGB, presently composed of 10 members, was founded in 2008 to coordinate Biobanks already supported as single core facilities by the Telethon Foundation. To date TNGB stores approx 80,000 samples and the catalogue lists about 750 genetic diseases.
The TNGB Charter sets out the principles agreed upon by the Partners, defines the organisation and governance as well as ethical guidelines, activities, policies, expected benefits, and duties. Governed by the Network Board, the TNGB is supported by a Coordinator Emeritus and by an Advisory Board which includes experts in legal, ethical and technical issues as well as a representative of UNIAMO, Federation of Patients' Associations.
Since its establishment, the TNGB has focused on improving operating procedures, implementing quality control systems, improving visibility and quality of the online catalogue access, pursuing outreach strategies to disseminate information about its services throughout the scientific community and associations of patients.
The TNGB interoperability has been greatly facilitated by adopting an IT infrastructure, which has been instrumental in helping the processes of harmonising and standardising all TNGB activities. The IT platform, managing the workflow of samples, not only generates a centralised, continuously updated online catalogue but also enables coordinated management and common rules for catalogue access based on a unique "Request Control Panel". This prevents depletions of valuable samples due to duplicate sample requests by a user to the individual Biobanks.
During the last 5 years, scientists and clinicians have extensively used TNGB services for different reasons, as shown by 249 papers acknowledging the TNGB. Patients/family-members, who needed samples from their affected index cases for genetic counselling and/or prenatal diagnosis, also made use of TNGB services. TNGB has also developed a relationship with Family Associations with the aim to offer a service to the patients' families as well as to centralise worldwide rare samples for specific research projects. In particular, 5 agreements between TNGB and Family Associations have been signed by the parties and by the Coordinator, on behalf of Telethon.
Another important TNGB's achievement has been the implementation of a cost recovery system aimed at partially recovering the cost of some basic procedures related to the TNGB distribution services.
TNGB is also involved, as associated member, in 2 European projects (BBMRI- and FP7 RD-Connect). Recently, TNGB has joined the EuroBioBank network, to which 5 Biobanks were already affiliated.
Finally, TNGB relies on the expertise of the European Centre for Law, Science and New Technologies, University of Pavia to review emerging ethical, legal and societal issues (ELSI) with regulatory bodies (Garante Privacy Authority).
General information and documents including the online catalogue are available on the TNGB website http://www.biobanknetwork.org/.

ABSTRACT N. 255

Telethon Research Services/Core Facilities		
Principal Investigator	**POLISHCHUK ROMAN**	
Telethon grant N.	**GTF08001**	
Total budget €	**183.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2009**

TELETHON ELECTRON MICROSCOPY CORE FACILITY (TEEMCOF)

Polishchuk Elena (1,2), Iacobacci Simona (1), Turacchio Gabriele (2), Polishchuk Roman (1)

(1) Telethon Institute of Genetics and Medicine, Via Pietro Castellino, 111, 80131, Naples, Italy, Tel. +39.081.6132336, Fax +39.081.5609877, E-mail: polish@tigem.it
(2) Institute of Protein Biochemistry, CNR, Naples, Italy

Studies of genetic diseases require many steps, from identification of the gene responsible for the pathology development, to discovery of therapeutic approaches for the disease treatment. The key step, clarification of the mechanisms of pathogenesis, needs a careful characterization of the molecular mechanisms involved in the physiology of such aberrant gene products (ie. its synthesis, turnover, interactions with components of molecular machineries, and precise cellular localization). At this stage, EM plays a fundamental role in the understanding of disease development, providing high resolution detection of anomalous gene products and analyses of ultrastructural changes induced by their expression. However, in contrast to genetic and molecular biology approaches, EM studies remain a dream for many laboratories due to high cost and lack of the necessary instrumentation and expertise.
The aim is thus to provide help for Telethon projects that require EM approaches for the analysis of genetic diseases. Long-standing expertise, technical knowledge, instrumentation and trained personnel are all available for such help at the Telethon Electron Microscopy Core Facility (TeEMCoF).
The TeEMCoF provided technical assistance and expertise, along with

qualitative and quantitative evaluation of EM data, for numerous studies supported by Telethon grants. It comprised the the effort of experienced scientists, technicians, and extensive use of equipment to effectively perform EM studies requested by Telethon scientists.
Therefore, with the help of Telethon and in collaboration with Telethon-supported scientists, we expanded further our understanding of functional genomics from the cellular to the whole-body level and helped to design new tools for the detection and treatment of genetic diseases.

ABSTRACT N. 256

Telethon Research Services/Core Facilities		
Principal Investigator	**PESOLE GRAZIANO**	
Telethon grant N.	**GTF09024**	
Total budget €	**105.000**	
Centres: **1**	*Duration (yrs):* **3**	*Starting year:* **2010**

WEP: AN HIGH-PERFORMANCE ANALYSIS PIPELINE FOR WHOLE-EXOME SEQUENCING DATA

Mattia D'Antonio (1), De Meo D'Onorio Paolo (2), Paoletti Daniele (2), Elmi Berardino (1), Pallocca Matteo (2), Sanna Nico (2), Picardi Ernesto (1), Pesole Graziano (1,3,4), Castrignanò Tiziana (2)

(1) Dipartimento di Bioscienze, Biotecnologie e Scienze Farmacologiche, Università degli Studi di Bari, via Orabona, 4, Bari, Italy, Tel. +39.080.5443588, E-mail: graziano.pesole@uniba.it
(2) CASPUR, Consorzio interuniversitario per le Applicazioni di Supercalcolo per Università e Ricerca, Rome, Italy
(3) Istituto di Biomembrane e Bioenergetica, Consiglio Nazionale delle Ricerche, Bari, Italy
(4) Center of Excellence in Genomics (CEGBA), Bari, Italy

Background
The advent of massively parallel sequencing technologies (Next Generation Sequencing, NGS) profoundly modified the landscape of human genetics.
In particular, Whole Exome Sequencing (WES) is a primary application of NGS that focuses on the exonic regions of the eukaryotic genomes; exomes are ideal to help us understanding high-penetrance allelic variation and its relationship to phenotype. A complete WES analysis involves several steps which need to be suitably designed and arranged into an efficient pipeline.
Managing a NGS analysis pipeline and its huge amount of produced data requires non trivial IT skills and computational power.
Results
Our web resource called WEP (Whole-Exome sequencing Pipeline web tool) performs a complete WES pipeline and provides easy access through interface to intermediate and final results. The WEP pipeline is composed of several steps:
1) verification of input integrity and quality checks, read trimming and filtering; 2) gapped alignment; 3) BAM conversion, sorting and indexing; 4) duplicates removal; 5) alignment optimization around insertion/deletion (indel) positions; 6) recalibration of quality scores; 7) single nucleotide and deletion/insertion polymorphism (SNP and DIP) variant calling; 8) variant annotation; 9) result storage into custom databases to allow cross-linking and intersections, statistic assessments and much more. In order to overcome the challenge of managing large amount of data and maximize the biological information extracted from them, our tool restricts the number of final results filtering data by customizable thresholds, facilitating the identification of functionally significant variants. Default values are also provided at the analysis computation completion, tuned with the most common literature work published in recent years.
Conclusion
Through our tool a user can perform the whole analysis without knowing the underlying hardware and software architecture, dealing with both paired and single end data. The interface provides an easy and intuitive access for data submission and a user-friendly web interface for annotated variant visualization.
Non-IT mastered users can access through WEP to the most updated and tested WES algorithms, ad-hoc tuned to maximize the quality of called variants while minimizing artifacts and false positives.
The web tool is available at the following web address: http://www.caspur.it/wep.

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FONDAZIONE TELETHON

Rome headquarter
Via Carlo Spinola, 16
00154 Rome, Italy
Tel. +39 06 440151
Fax +39 06 44202032

Milan headquarter
Piazza Cavour, 1
20121 Milan, Italy
Tel. +39 02 2022171
Fax +39 06 44015600

www.telethon.it e-mail: telethon@telethon.it