ISTITUTO SUPERIORE DI SANITÀ

Workshop. **Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions**

Istituto Superiore di Sanità
Rome, October 29-31, 2008

ABSTRACT BOOK

Edited by
Domenica Taruscio and Marco Salvatore
Centro Nazionale Malattie Rare

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The International Conference on rare diseases and orfan drugs (October, 27th-31st, 2008) is an annual meeting aimed to illustrate Italian and European activities as well as the new developments concerning rare diseases and orphan drugs. The first two days of the meeting (27 and 28 October) will be dedicated to the presentation and discussion of the main European and Italian activities on rare disease and orphan drugs; from the 29 to the 31 of October, during the second workshop entitled "Projects on rare diseases funded within the bilateral agreement Italy (Istituto Superiore di Sanità) and USA (NIH, Office for Rare Diseases) on joint research and development of public health actions", the results of the research projects, funded within the bilateral agreement (Italy-USA) on joint research and development of public health actions between ISS and NIH-Office for Rare Diseases will be presented. In this abstract book, we collected all the scientific contributions from the scientific responsible of each project funded.

Key words: Rare diseases, Orphan drugs, Research

Istituto Superiore di Sanità
Workshop. Progetti sulle malattie rare finanziati nell'ambito dell'accordo bilaterale fra Italia (Istituto Superiore di Sanità) e USA (NIH, Office for Rare Diseases) sulla ricerca scientifica e lo sviluppo di iniziative di sanità pubblica. Istituto Superiore di Sanità. Roma, 29-31 ottobre 2008. Riassunti.
A cura di Domenica Taruscio e Marco Salvatore
2008, xvii, 154 p. ISTISAN Congressi 08/C10 (in inglese)

La Conferenza Internazionale sulle malattie rare ed i farmaci orfani (27-31 ottobre, 2008) rappresenta un appuntamento annuale durante il quale vengono illustrate le attività intraprese a livello naizonale nel settore malattie rare e farmaci orfani e proposte possibili nuove iniziative tenendo conto anche del più ampio contesto europeo. Nei primi due giorni della Conferenza (27-28 ottobre) ampio spazio sarà dedicato alla presentazione ed alla discussione delle principali novità europee ed italiane sulle iniziative ed attività nel campo delle malattie rare e farmaci orfani. Negli ultimi tre giorni della Conferenza (29-31 ottobre) si svolgerà il secondo workshop finalizzato a presentare i risultati raggiunti nell'ambito dei progetti finanziati nel contesto dell'accordo bilaterale Italia-USA (ISS/NIH-Office for Rare Diseases). Nel presente volume sono stati raccolti tutti gli abstract elaborati da ciascun Responsabile di progetto e presentati nel corso del workshop.

Parole Chiave: Malattie rare, Farmaci orfani, Ricerca

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La responsabilità dei dati scientifici e tecnici è dei singoli autori.

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Enrico Garaci
President of the Istituto Superiore di Sanità, Rome, Italy
Stephen Groft
Director of the Office for Rare Diseases, National Institute of Health, Bethesda, MD, USA

Session I
ASPECTS OF PATHOGENESIS

Chairpersons: **Stephen Groft, Domenica Taruscio**

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Thuersday, October 30, 2008

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Friday, October 31, 2008

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Chairpersons: **Adriana Albini, Stefano Vella**

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17.15 *Combined treatment with statins and aminobisphosphonates in mandibuloacral dysplasia fibroblasts*
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17.15 *Enzyme replacement therapy with alglucosidase alfa in juvenile-adult glycogenosis type 2 patients*
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Armando Gabrielli, Giovanni Pomponio, Paolo Fraticelli, Michele Luchetti, Silvia Svegliati, Gianluca Moroncini, Roberto Giacomelli, Paola Cipriani, Alessandra Marrelli, Vasiliki Liakouli, Elisa Pingiotti, Vincenza Dolo, Danilo Millimaggi, Sandra D'Ascenzo, Ilaria Giusti, Serena Guiducci, Marco Matucci-Cerinic, Sergio Generini, Gianfranco Ferraccioli, Barbara Tolusso, Maria De Sanctis, Walter Malorni, Anna Maria Giammarioli, Elisabetta Straface, Marina Pierdominici, Angela Maselli, Laura Somma, Serena Vettori, Giuseppina Abignano, Gabriele Valentini, Patrizia Rovere-Querini, Stefano Franchini, Angelo Andrea Manfredi, Maria Grazia Sabbadini

18.00 *Testing* in vitro *and* in vivo *treatments for inclusion body myositis*
Simona Saredi, Claudia Di Blasi, Pia Bernasconi, Lucia Morandi, Renato Mantegazza, Marina Mora, Cristina Sancricca, Enzo Ricci, Pietro Attilio Tonali, Massimiliano Mirabella

18.15 Discussion and general remarks

NOTES FOR READERS

This abstract book presents the oral and poster presentations illustrated during the three days Workshop (29th-31st, October, 2008) organized in the frame of the annual international Conference on rare diseases and orphan drugs (Istituto Superiore di Sanità, 27th-31st October, 2008). The abstracts are listed in alphabetical order based on the first author of the contribution. Posters are indicated by the letter "P" before the title. All authors of the abstracts are listed at the end of the volume, in the specific "author index".

The programme of the Workshop is included; all abstracts are presented as oral or poster presentations.

Communications and Posters

ANGIOGENESIS AND INFLAMMATION AS TARGET FOR RETINOBLASTOMA THERAPY

Adriana Albini (a), Rosaria Cammarota (a), Roberta Venè (b), Gianfranco Fassina (b), Massimo Nicolò (c), Douglas M. Noonan (d), Francesca Tosetti (b)

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(c) Dipartimento di Neuroscienze, Genetica e Oftalmologia, Sezione Clinica Oculistica, Università degli Studi, Genova

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Introduction. Ocular tumors, in particular retinoblastoma and uveal melanoma, are rare diseases but with a very high impact on patients. The conventional chemotherapy is toxic so our aim was to identify new molecular targets to restrain tumour progression without damages for patients. Both are highly vascular and appear to be readily targeted by anti - angiogenic agents.

Methods. The sensitivity of Y79 cells to different prooxidant anticancer drugs including the synthetic retinoid N-(4-hydroxyphenyl)retinamide (4HPR), arsenic trioxide (As_2O_3) and phenetyl isothiocyanate (PEITC) was assessed by the MTT assay, ATP content quantification and release of extracellular lactate dehydrogenase Survival signaling stimulated by the Insulin-like Growth Factor I (IGF-I) and cell death pathways were analyzed in prooxidant-treated cells *in vitro* and correlated with proangiogenic IGF-1 signaling in the matrigel sponge model assay *in vivo*. To further examine the potential of targeting the microenvironment as a strategy to control tumor growth, we adopted a gene transduction approach using a potent TH1 cytokine endowed with strong anti-angiogenic activity, Interleukin-12 (IL-12). Gene transfer into murine 99E1 ocular tumor cells, while having no effects on growth *in vitro*, essentially blocked growth of vascular tumors *in vivo* without evident signs of toxicity. The 99E1 cell line was derived from a choroidal/retinal pigmented epithelial ocular tumor that arose in a transgenic FVB/N mouse bearing the SV40 oncogene.

Results. In Y79 cells treated with 4HPR where IGF-I-induced AKT phosphorylation was repressed, phosphorylation at Ser 9 of the multifunctional kinase glycogen synthase kinase 3β (GSK3β) was sustained. All the prooxidant drugs investigated were able to induce GSK3β phosphorylation, which was reversed by chemically different antioxidants, concomitant with mitochondrial and nuclear apoptosis and ATP depletion. The *in vivo* angiogenesis assay revealed that IGF-1 receptor stimulation remarkably contributes to the proangiogenic potential of retinoblastoma cells. Orthotopic intraocular injection resulted in invasive tumors that destroyed ocular architecture by the control cells while the IL-12 transduced cells rarely formed tumors. Histological analysis revealed highly invasive and angiogenic tumor growth in the controls and poorly vascularized tumors in the presence of IL-12. The tumor repression effect could be reproduced by a systemic anti-angiogenic effect, where controlateral injection of IL-12 expressing cells strongly repressed growth in

tumors formed by parental 99E1 cells. This was associated with significantly lowered tumor vessel densities, a trend towards lower VEGF levels in the lesion, and significantly decreased NK cells in the parental tumors exposed to systemic IL-12.

Conclusions. Prooxidant anticancer drugs interfere with retinoblastoma survival and proangiogenic signaling pathways thus suggesting potential application at clinical level. IL-12 gene transfer can provide anti-angiogenic effects without toxicity and may be particularly suited for therapy of vascularized ocular tumors.

DEVELOPMENT OF NEW STRATEGTIES OF MOUSE AMELANOMA VACCINATION USING L19MTNFA AS AN ADJUVANT

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The antitumor effects of the pro-inflammatory cytokine TNFα are mainly due to the preferential toxicity for the tumor-associated vasculature and to the ability to potentiate the immune response against tumors. For these reasons, TNFα could be used as an adjuvant in the formulation of antitumor vaccines.

L19mTNFα is a fusion protein composed by the scFv L19, specific for the highly conserved ED-B domain of fibronectin, a tumor-associated antigen identical in humans and mice, and m(ouse)TNFα that, in different mouse tumor models, induces a therapeutic T cell-mediated immune response that protects the host against syngeneic tumors of different histological origin.

The purpose of this study was to evaluate the efficacy of L19mTNFα as an adjuvant in vaccination protocols with melanoma homogenates.

Two melanoma models (B16F1 and B16BL6B17) were established in the syngeneic C57 black mice. These melanomas are low immunogenic tumors that grow fast and do not respond to the systemic therapy with L19mTNFα and melphalan that, on the contrary, cures 80% of WEHI-164 fibrosarcoma- and 20-30% of C51 colon carcinoma-bearing mice.

Mice were *s.c.* injected at 3 week intervals with melanoma homogenate (80 mg/mouse) with or without the addition of L19mTNFα (1 μg/mouse). Three weeks after the last vaccination, a tumorigenic dose of melanoma cells was *s.c.* inoculated and the tumor growth was recorded.

Up to three injections of tumor homogenate, supplemented or not with L19mTNFα, determined no significant B16F1 or B16BL6B17 tumor growth delay in all the vaccinated groups respect to controls. Moreover, the treatment with L19mTNFα and melphalan didn't induce valuable therapeutic advantages in the vaccinated mice respect to controls. On the contrary, after four injections, the mice vaccinated with tumor homogenate supplemented with L19mTNFα developed palpable tumors one week after the controls and three days after the mice vaccinated with homogenate only. Moreover, in the same group of animals the therapy with L19mTNFα and melphalan determined a reduction in the tumor growth rate that almost doubled the life-span expectancy. Noteworthy, after the third injection, all the mice vaccinated with homogenate supplemented with L19mTNFα presented patches of alopecia areata and hair depigmentation usually associated to autoimmunity and described in mice actively immunized in the presence of adjuvants.

SYSTEMATIC DIAGNOSIS OF RARE ERYTHROENZYMOPATHIES: GENERATION OF GUIDELINES AND STUDY OF THE GENOTYPE/PHENOTYPE CORRELATION

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Erythroenzymopathies are a group of rare hereditary haemolytic anaemias caused by abnormalities in the genes encoding for the enzymes of the three main metabolic erythrocyte pathways: glycolysis, pentosephosphate shunt and nucleotide metabolism. The degree of haemolysis is variable and depends on the metabolic cycle involved, the relative importance of the affected enzyme, and the properties of the mutant enzyme with regard to kinetic abnormalities and/or instability. The ability to compensate for the enzyme deficiency by overexpressing isoenzymes or using alternative pathways contributes to the variability of clinical picture.

Moreover, if the defective enzyme is not confined to the red cells but also expressed in other tissues, non-haematological symptoms may occur. Defects of ubiquitous enzymes may cause prenatal mortality and therefore be rarely seen by clinicians.

In the first year of the project we focused on the study of the genotype/phenotype relationship in rare erythroenzymopathies. In particular, we investigated the clinical and molecular characteristics of 6 new patients with recessive hereditary methemoglobinemia due to cytochrome b5 reductase deficiency. One patient was affected by Type-II disease with cyanosis and severe progressive neurological dysfunction, whereas the others displayed the benign Type-I phenotype.

Eight different mutations were detected among the twelve mutated alleles identified, two of them (Gln27STOP and Arg45Trp) were new. Moreover, we characterized at the protein level three newly described missense mutations (c.187T>C, c.469G>C and c.740T>C) identified in patients with hemolytic anemia due to pyrimidine-5'-nucleotidase deficiency. The mutant enzymes (C63R, G157R and I247T) were obtained as recombinant forms by means of heterologous expression systems and site-directed mutagenesis techniques, and purified to homogeneity. The enzymes were altered, although to a different extent, in both thermal stability and catalytic efficiency, providing evidence that all affected aminoacids are functionally and structurally important for preserving the enzyme activity during the red cell life span. During the second year of the project we will elaborate guidelines for diagnostic and therapeutic approach to rare chronic hemolytic anemias due to defects of red cell metabolism. Firstly, we will review all diagnostic laboratory tests to create the updated reference intervals from normal individuals divided for age. We will also

consider normal reference intervals from a group of neonates because in some cases enzymatic activity could be different from adults causing inappropriate diagnosis.

Subsequently, we will review all diagnostic pathways of hemolytic anemias, and a flow chart will be created for each type of erythroenzymopathy, both considering diagnostic and therapeutic aspects. This will permit to extend the knowledge on patho-physiological and clinical aspects of RBC enzymopathies by a close cooperation of Experts and Expert Centres and it will facilitate clinicians in rapid identification of red blood cell enzyme defects and in appropriate treatment.

P. GENOTYPE/BEHAVIOURAL PHENOTYPE CORRELATIONS IN CORNELIA DE LANGE SYNDROME

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Cornelia de Lange Syndrome (CdLS [OMIM 122470]), is a rare multiple congenital anomalies/mental retardation syndrome with high phenotypic variability. Various behavioural phenotypes have been associated with CdLS especially involving self injurious, aggressive and self restraining behaviour and autism (-like) features.

Two different genes were identified as involved in CdLS: NIBPL and SMC1L1. Mutation in a third gene, SMC3, has been described in one patient. Up now global detection rate of molecular analysis in CdLS patients gives results of about 55-60% The correlation between genotype and phenotype are still in progress. Some preliminary results seems to identify more severe phenotypic features in NIPBL mutated patients also if this is not totally true at individual level.

To improve the available knowledge on the genotype/behavioural phenotype correlations in CdLS for different type of genetic defect.

Evaluation of cognitive functions and behavioral assessment was carried out in a group of clinically diagnosed CdLS patients by the following instruments: Wechsler Scales (WPPSI -R, WISC-R, WAIS) or Leiter International Performance Scales Revised (Leiter-R), Developmental Behavior Scale Primary Carer Version (DBC-P); Childhood Autism Rating Scale (CARS).

All of them were characterized at genetic level with mutational analysis for NIPBL and SMC1L1 genes and CGH-array analysis.

Among 28 CdLS patients 15 (53.6%) showed a mutation in NIPBL gene, 3 (10.7%) mutations in SMC1L1 gene; in the last 10 no genetic anomaly was found.

Mental retardation affected 85.7% of studied subjects (24 of 28); intellectual disabilities were mild in one case, moderate in 5, severe in 9 and profound in 9.

3/4 of CdLS subjects with borderline mental development don't show any genetic abnormality while in one patient a NIPBL mutation was found.

Severe behavioural problems were found in about one third of cases (9 of 28). No specific genotype/phenotype correlations emerged since mutations in NIPBL (3 cases), in SMC1L1 (2 cases) and no defect (4 cases) were found. A diagnosis of autism was point out in 7 out of 28 (25%) CdLS subjects. NIPBL mutations were present in a large percentage (71%) of them.

Our preliminary results confirm the difficulty in establish clear correlation at individual level between genotype and neuro-behavioural phenotype. Moreover the great majority of CdLS patients with borderline mental development don't show any known genetic defect while subjects with a diagnosis of autism show an high percentage of NIPBL mutations.

P. A REPRODUCTIVE RISK QUESTIONNAIRE IN FAMILIES WITH A CHILD AFFECTED BY CORNELIA DE LANGE SYNDROME

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Cornelia de Lange Syndrome (CdLS [OMIM 122470]), is a rare multiple congenital anomalies/mental retardation syndrome with high phenotypic variability. Up to now three genes, Nipped B Like (NIPBL), SMC1L1 and SMC3 respectively localized on the short arm of chromosome 5, X and on the long arm of chromosome 10, have been identified as candidate genes in Cornelia de Lange syndrome.

This recently discovered genetic heterogeneity produced the need to inform families (parents, brother and sisters of affected individuals) about different reproductive risk transmissions.

The availability in our Institution of a large cohort of CdLS patients have suggested the creation of a questionnaire as a tool to collect more data about the specific needs of their families. This questionnaire was formed of 24 items including the following sections: data about parents, data about severity of the clinical picture of the proband, understanding of genetic basis of the syndrome, knowledge and access to genetic testing, significance of test results according to reproductive decisions.

Up to now data are available from 28 families in which 12 females and 16 males CdLS probands were respectively present. All families had been given previously counselling about the diagnosis and clinical consequences.

Mean age of probands was 10.46 years (range 2-35 years). Mean age at CdLS diagnosis was 1 year (range at birth-12).

In all the probands a developmental delay was reported and in the great majority the level judged by the parents was mild to moderate. Proband's progresses and physician's help have contributed in improvement in life style of parents.

23/28 families were offered genetic counselling. Understanding of reproductive risk and anxiety concerning it showed little improvement after the session. 15/28 parents a second genetic counselling at time of subsequent pregnancy. Options for prenatal diagnosis selected by parents were amniocentesis and prenatal ultrasound scan. Opinion about efficacy of prenatal diagnosis was influenced by personal belief and educational status. In most cases there was a scanty knowledge about the limit of fetal ultrasound and amniocentesis.

In our preliminary results an apparent discordancy was present in confidence in genetic test application to define parents and offsprings reproductive risk, proband's prognosis and application in future therapy.

These results show a lack of knowledge about the genetic heterogeneity of the syndrome by the families up to now evaluated the need of revaluation of the inheritance patterns and risk estimation, applying the new knowledge about the aetiology of this disease.

TRAUMA AND RISK OF AMYOTROPHIC LATERAL SCLEROSIS

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There is no valuable evidence on the role of environmental risk factors in patients with Amyotrophic Lateral Sclerosis (ALS). Traumatic events (single or repeated) have been quite extensively investigated and several studies showed traumatic events being present to a greater extent in the history of patients with ALS than in the general population. However this evidence is still controversial because of methodological issues (recall bias, referral patients, under-ascertainment of trauma). The aims of the study are: whether or not ALS patients are at increased risk of trauma, repeated trauma, severe trauma; whether the site of the major trauma coincides with the site of onset of symptoms; to verify the consistency of the association in subgroups of patients from different geographic areas.

This population based case-control study includes three Italian regions (Lombardia, Piemonte, Puglia) with population-based registries enrolling newly diagnosed residents with ALS. Each patient diagnosed since January 1 2007 is matched for age, sex and residency to two hospital controls (neurological and non neurological).

For each patient demographic and clinical data are collected. The diagnosis is made according to the El Escorial criteria (original & revised). The consistency of the data is tested in each regional registry separately and in multivariate analysis models adjusting for the main confounders (professional activity, alcohol intake, smoking, exposure to potentially toxic agents, family history of degenerative disorders).

Based on literature records, about 1% of individuals in the general population reports a history of severe traumatic events. If ALS carries a 4-fold risk of traumatic events, a total of 410 patients and 819 controls must be recruited in 2 years (alpha 0.05; beta 0.8). The study is also powered to detect a 5-fold risk of trauma by recruiting 244 ALS patients and 488 matched controls.

As of July 1 2008, a total of 105 patients (male 56.2%; mean age 66 years) and 91 matched controls were recruited. According to the revised El-Escorial criteria, 38 patients had definite ALS, 20 had probable ALS and 6 had possible ALS (diagnostic category non specified in 41 cases). 51 patients with ALS (48.6%) with data available reported a history of at least one traumatic event (35 controls; 49.3%) (*Odds Ratio* 1.3, 95% CI 0.7-2.4).

GASTROESOPHAGEAL REFLUX IN SYSTEMIC SCLEROSIS: RELATIONSHIP WITH PULMONARY INVOLVEMENT

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Background. Esophageal disease, a frequent complication of Systemic Sclerosis (SSc), is characterized by impaired motility and Gastroesophageal Reflux (GER). GER has been associated with interstitial lung disease, but its pathogenetic role is still debated. Esophageal pH monitoring is at present considered the gold standard for the diagnosis of GER, however it detects only acid events; pH-multichannel intraluminal impedance/24 h (pH-MII) is a new diagnostic tool for the evalutation of gastroesophageal disease and detects the nature and the pH of the refluxate (acid, weakly acid, non-acid).

Aims of the study. i) To assess the pH properties of the gastroesophageal refluxate in SSc; ii) to evaluate whether a relationship exists between type of reflux and pulmonary involvement; iii) to identify patients with GER at risk for lung disease.

Patients and Methods. Eleven females (aged 59±14 yrs), who fulfilled the American College of Rheumatology criteria for SSc, were enrolled. Antisecretory therapy was withdrawn 15 days before the procedure, which consisted of esophagogastroduodenoscopy followed by pH-MII/24h monitoring. High Resolution chest Computed Tomography (HRTC) and pulmonary function tests (spirometry and diffusing capacity for carbon monoxide, DLCO) were used to assess lung involvement.

Results. All patients had GER assessed by pH-MII, and the majority (55%) had weakly acid refluxate; acid refluxate was detected in 2 patients (18.2%), non-acid in 3 (27.3%). Only 2 patients (1 with non-acid and 1 with weakly acid refluxate) were symptoms-free. All patients had lung disease (any among HRTC, spirometry, DLCO). No correlation was found between the type of refluxate and lung disease; however patients with acid GER less frequently had abnormal HRTC (17% *vs* 34% *vs* 50%, acid *vs* non-acid *vs* weakly acid) and DLCO<80% (14% *vs* 29% *vs* 57% acid *vs* non-acid *vs* weakly acid). Anticentromere Antibodies (ACA) were found in 100% of acid GER patients, in 71% of weakly acid GER patients and in none of non-acid GER patients.

Conclusions. Patients with non-acid refluxate without ACA seem to be at risk of pulmonary involvement. These preliminary data need to be confirmed in a larger cohort of SSc patients.

ENZYME REPLACEMENT THERAPY WITH ALGLUCOSIDASE ALFA IN JUVENILE-ADULT GLYCOGENOSIS TYPE 2 PATIENTS

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Background. Glycogenosis 2 (G2), a lysosomal storage disorder due to Acid Alfa-Glucosidase (GAA) deficiency, has recently demonstrated to be responsive to human recombinant (rhGAA) Enzyme Replacement Therapy (ERT) in infantile phenotypes. To date, no study has been published on juvenile-adult phenotypes. An independent multi-centric Italian study, supported by the Agenzia Italiana del Farmaco, was proposed in 2006 to verify ERT effectiveness in late-onset phenotypes.

Methods. 29 patients (13 females, 16 males; 7 children, 22 adults) were enrolled; they received a bi-weekly infusion of 20 mg/kg of rhGAA, and were monitored for: general conditions, respiratory function, ventilatory support, 6-Minute Walking Test (6MWT), Walton Scale (WS), muscular imaging. Biochemistry included: CK, LDH, AST, ALT, blood pCO2.

Results. After 12 months of ERT, tracheotomy was removed in 3 of the 4 carrying patients (not further ventilatory support was needed in 2); mean ventilation time decreased from 15.5 to 9.6 hours. All patients improved in their 6MWT ($p<.0001$) and WS severity decreased in 5 patients (p 0.0039). Headache and muscle pain respectively affecting 27.6% and 37.9% of patients at baseline persisted in 6.9% and 10.3% of them after one year. A statistically significant reduction in muscular enzyme levels was observed: CK (p 0.0075), LDH (p 0.0003), AST (p 0.0028), ALT (p 0.0023). No patient showed secondary effects to ERT.

Conclusions. rhGAA therapy demonstrated to be safe and capable to improve clinical and laboratory symptoms in treated patients. Results showed to be independent of patient age and disease severity.

PRECLINICAL STUDIES AIMED TO DEVELOP TARGET GENES-BASED THERAPIES FOR THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease still orphan of a truly effective therapy. ALS is characterized by the selective loss of motoneurons, leading to muscular atrophy and progressive paralysis, and culminating in death of patients within 2-5 years after diagnosis. It is emerging evidence that in the motoneurons of patients with sporadic ALS and in animal models of the disease, there is a remarkable activation of pro-degenerative pathways (like p38 MAP-Kinase). On the other side, the mechanisms involved in the modulation of cell survival (like PI3K/Akt pathway) are not activated, thus suggesting an impairment in the induction of neuroprotective responses.

These pathways may be considered as potential therapeutic targets. However, bioavailability to central nervous system and cell-specificity of any treatment is still a great challenge.

Based on these evidences, with this project we propose: i) to develop gene-targeted strategies aimed at counteracting p38 pro-degenerative pathway and activating Akt pro-survival cascade inside motorneurons of spinal cord; ii) to evaluate efficacy and safety of these potential therapeutic interventions in an animal model of ALS.

We have developed lentiviral vectors expressing candidate p38-targeted shRNA sequences. These shRNAs were tested in primary mouse astrocyte-motoneuronal cell co-cultures or in cultures of rat cortical neurons. They were able to prevent activation of p38 and its downstream targets after TNFalpha stimulation, and to reduce neuronal loss after toxic stimuli. In parallel, we have developed constructs that express constitutively active forms of Akt1 and Akt3 (caAkt1 and caAkt3). Preliminary experiments in cell lines showed that either caAkt1 or caAkt3 efficiently phosphorylates and inhibits downstream pro-apoptotic targets, such as GSK3beta. However, when we tested the same constructs in primary mouse astrocyte-motoneuronal cell co-cultures, we observed that caAkt1 exerts mainly a pro-proliferative function on astrocytes, leading to alterations in their morphology. caAkt3, instead, induced increased arborisation in neuronal cells, suggesting a potential trophic effect in this cell type.

Based on these evidences, we hypothesized that expressing caAkt3 under the control of a neuronal-specific promoter could be instrumental in the induction of pro-survival and regenerative responses in motoneurons.

Furthermore, we are aimed to deliver p38-targeting shRNAs and caAkt3 constructs in the spinal cord of an ALS mouse model and to monitor their potential efficacy on motor performances and disease progression.

GENOMIC DIAGNOSIS AND CLASSIFICATION OF RARE DISORDERS WITH MENTAL RETARDATION USING HIGH THROUGHPUT TECHNOLOGIES

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Copy Number Variations (CNVs) represent a major cause of Mental Retardation (MR), especially when it is associated to Multiple Congenital Anomalies (MCA). In the last few years, DNA microarrays have increased capabilities for detection of cryptic pathogenic CNVs by analyzing the entire genome. Several commercially platforms, including a large number of spotted sequences, oligonucleotides or Single-Nucleotide Polymorphisms (SNPs), are now available to study the whole genome at a high resolution.

We have examined during last year 204 subjects with MR using an oligonucleotide-array with an average resolution of 75 Kb, and disclosed 35 pathogenic CNVs in 33 patients (16%), spanning 0.208-22.473 Mb. These results confirmed the oligonucleotide array-CGH to be a powerful tool in the study of MR. Forty MR/MCA patients, selected from the original cohort of 204 subjects, were re-run on a commercial SNP-array platform, consisting of about 250,000 SNPs with an average spacing of 10 Kb. Twenty eight resulted negative with oligonucleotide-array analysis, while 12 were positive (30%). This analysis allowed to compare the robustness and the cost/effectiveness of the two platforms in detecting pathogenic cryptic genomic changes. SNP-array analysis detected 24 CNVs in 16 different patients (40%), spanning 0.152-23.022 Mb. Nineteen of the 24 CNVs were confirmed with other techniques, whereas 5 changes were considered false positives (21%).

Fourteen of the confirmed CNVs were completely overlapping (74%) when the oligonucleotide and SNP data were compared. In both analyses 8 patients showed a deletion, 1 had two non-contiguous duplications on the same chromosome, while 2 had a deletion and duplication on a single chromosome. These changes are assumed to be pathogenic, based on their extent and the genes involved. SNP array results disclosed 5 CNVs not detected by array-CGH, including 3 deletions and 2 duplications, ranging in size from 0.152 to 0.393 Mb, confirming that the higher density array is more sensitive in detecting smaller CNV regions.

However, 3 of these CNVs did not contain known genes, questioning their pathogenic role. In conclusion, the two tested platforms have proved to have good performance in detecting possible causes of MR. Although the higher resolution of SNPs-array is capable of deciphering disorders caused by small imbalances involving single genes, the higher rate of false-positive results and the higher percentage of CNVs devoid of any clear pathogenic role suggest a prudent use of this technique in the diagnostic practice.

NEUROFIBROMATOSIS TYPE 1: DEVELOPMENT OF A NOVEL PROGRAM FOR MOLECULAR DIAGNOSIS AND CLINICAL FOLLOW-UP

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Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder due to mutations in the NF1 gene and characterised by *Café-au-Lait* Spots (CLS), inguinal/axillary freckling, neurofibromas, iris hamartomas (Lisch nodules), optic gliomas, and distinctive osseous lesions. These signs, however, are age-dependent, and present high variability in penetrance and expressivity even between affected members of a family. In addition, mutation detection in the NF1 gene is complex, time-consuming and expensive due to the large size of the gene, the presence of pseudogenes, the lack of hot spots, and the great variety of possible mutations.

Hence, the clinical and molecular diagnosis of NF1 may be still a challenge. In light of these considerations, we have investigated 38 adults (≥18 years) for mutations in the NF1 gene using denaturing High-Performance Liquid Chromatography (dHPLC)/sequencing: twenty-three of them presented with a clinically-diagnosed NF1; the remaining 15 presented at least one clinical feature of NF1, but they did not meet the NIH diagnostic criteria in spite of the adult age. Notably, NF1 mutations were detected in only 13 of 23 (~55%) of the clinically-diagnosed patients and in 2 of 15 (~13%) of the others. These data further support the need for different techniques to detect the complete diversity of NF1 mutations, and suggest that patients harbouring NF1 mutations may sometimes not fulfil the NIH criteria.

Therefore, a more structured program has been developed to characterize more precisely the clinical spectrum, natural history, gene mutations, prognosis and follow-up of patients (including adults) with NF1 and NF1-like entities (*e.g.*, isolated CLS). At baseline, patients with at least one sign of NF1 will be evaluated by well-skilled and broadly experienced specialists, including a neurologist, dermatologist, and ophthalmologist; contrast MRI of the brain and spine will be performed to look for intraspinal tumours and curvature of the spine; chest-X-ray and abdominal ultrasonography to look for intrathoracic and abdominal tumours (*e.g.*, pheochromocytomas); visual evoked potentials for optic gliomas (in addition to visual acuity and visual fields); a neuropsychological battery that we have recently developed, will be performed for cognitive and affective evaluation. SALSA MLPA assay will be implemented, trying to increase the detection rate of mutations in the NF1 gene.

Thereafter patients will be clinically evaluated by at least one experienced neurologist trained in NF1 every 6 or 12 months; visual acuity, fields and VEPs in children will be performed every 6-12 months; MRI will be obtained every 5 years, also in absence of novel signs (*e.g.*, radicular pain, visual disturbances, etc.).

The program will start as of September 2008.

P. CYTOKINE-BASED IMMUNOTHERAPY AND SUBVERSION OF TUMOR-RELATED IMMUNOSUPPRESSION IN CUTANEOUS AND OCULAR MELANOMA MODELS

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Ocular melanoma is a rare disease, with distinct clinical and biological features from the more frequent cutaneous form as it develops predominantly liver metastases. Clinical response rates to chemo or immunotherapy in metastatic melanoma have limited impact on survival rates. We studied the development of new cytokine-based vaccine treatments combined with antibodies blocking CD4+CD25+Foxp3+ regulatory T cells (Treg) in pre-clinical models of metastatic melanoma.

The B16LS9 melanoma subline was previously derived from B16 cutaneous melanoma by repeated passages of splenic injection and collection of liver metastases (LS=liver/spleen). B16LS9 retain metastatic tropism for the liver when injected *i.v.* or in the posterior eye chamber. To target GM-CSF to melanoma cells, we engineered GM-CSF with an RGD peptide, which binds to the alpha-V/beta-3 integrins, with the aim to enhance local immune-stimulating effects.

The GM-CSF ORF was chimerized at its 3' end with a synthetic RGD-encoding sequence and cloned in an expression plasmid (pcDNA3.1hygro-RGD-GM-CSF), which was transfected into B16F10 melanoma cells.

Transfected cells released biologically active GM-CSF/RGD (10ng/ml/24h/10^6 cells), as detected by a proliferation assay on the GM-CSF-sensitive BAF3/GM subline. Part of the GM-CSF/RGD was also displayed on the cell membrane of melanoma cells, as detected by immunofluorescence with an anti-GM-CSF antibody.

Transfected B16F10 cells were then used as adjuvants by admixing them with B16LS9 cells as cellular vaccine for the immunotherapy of mice bearing B16LS9 metastases. By *i.v.* injection, 100% of control mice developed lung and liver metastases within 25 days (mean survival time 20.7±2.3), indicating that B16LS9 is a highly aggressive tumor. Immunotherapy by administration of the irradiated cellular vaccine at day +2, +4, +7 +10, +14 after initial tumor challenge induced only a slight increase in mean survival time (22.9±4).

Therefore we speculated that the efficacy of the vaccine could be limited by either pre-existing or tumor-induced Treg cells.

We then tried to combine this treatment with an anti-CD25 mAb (PC61, 0.5mg per mouse), which targets Treg cells. Although the administration of anti-CD25 antibody alone had no effect on tumor-free survival of mice bearing B16LS9 metastatic disease, when it

was combined with the cellular vaccine a highly significant increase in tumor-free survival was observed (>41.1+9, P<0.0001), with about 30% of mice surviving for more than 45 days after primary challenge. We are now investigating other combinations which may enhance the effectiveness of the vaccine, such as immunomodulating anti-OX40 mAb or a TNF-alpha-scFv antibody targeting the melanoma extracellular matrix.

PUTATIVE ROLE OF MITOCHONDRIA IN THE PATHOGENESIS OF SPINOCEREBELLAR ATAXIA TYPE 1 (SCA1)

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SCA1 is an autosomal dominant inherited disorder characterized by degeneration of cerebellar Purkinje cells and spinocerebellar tracts owing to the expansion of an unstable CAG repeat. SCA1 stable transgenic mice expressing human SCA1 gene with either a normal or an expanded CAG tract have been generated. While all transgenic lines expressing the unexpanded SCA1 allele had normal behaviour, transgenic animals expressing the PS-82 transgene (hetero- and homozigous) developed ataxia and displayed an abnormal neurological phenotype.

Multiple lines of evidence suggest that neuronal death in SCAs might result from the direct involvement of mitochondria in the pathogenesis of these diseases. So we planned to study the mitochondrial oxidative enzymatic activity and the mitochondria-mediated apoptosis in SCA1 transgenic mice. The extent of Purkinje cell dendritic harborization was assessed by Haematoxylin-Eosin (HE) staining and cell death was analyzed performing TUNEL staining on cerebellar sections.

To study the mitochondrial enzymatic activities, cryostat sections of cerebella were histochemically stained for COX and respiratory chain enzyme activities were measured on cerebellar homogenates.

Finally, to evaluate putative modifications in expression level of several apoptotic proteins, Western blot analysis were performed on cerebellar homogenates.

All the analysis were done on cerebella from wt, hetero- and homozygous mice taken at different time-points during the developing of the disease.

At 2 months, at ultrastructural level, a slight loss of the Purkinje cell population and some ectopic PuC were found in the molecular layer of homozygous mice cerebella while these changes were milder in heterozygous ones. In both 6 month-old mice these effects were more severe. In addition, TUNEL-positive cells were observed in 2 month-old mice, more consistently in homozygous than in heterozygous mice. The number of apoptotic PuC increased in 6 month-old mice.

COX histochemistry showed a pronounced decrease of COX-specific signal in homozygous mice both at 2 and at 6 months, and milder decreases in heterozygous ones. Ultrastructural enzymatic COX examination confirmed these data whereas enzymatic assay of respiratory chain activities performed on homogenates did not show generalized defects.

Finally, immunoblottings revealed a gradual upregulation of Bax and a downregulation of Bcl-2 protein levels in hetero- and homozygous cerebellar homogenates during time.

We found a severe cytochrome-c-oxidase deficiency in PuC of SCA1 mice that interestingly parallels the severity and the progression of the disease during time. Moreover, this oxidative stress contribute to trigger the mitochondrial apoptotic pathway leading to specific Purkinje cell death.

TRANSCRIPTIONAL STUDY OF P63ALPHA MUTANTS FOUND IN ECTODERMAL DYSPLASIA SYNDROMES

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The Ectodermal Dysplasia (ED) syndromes are a group of inherited autosomal dominant human diseases. The prototypic Ectrodactyly, Ectodermal dysplasia and Cleft lip/palate (EEC) syndrome is clinically characterised by ectodermal dysplasia affecting skin, hair, nails teeth and facial clefts. Heterozygous mutations in the p63 gene have been identified in EEC syndrome patient and in other ED syndromes, including Ankyloblepharon-Ectodermal Defect-Cleft Lip and/or Palate (AEC), Limb-Mammary Syndrome (LMS), Acro-Dermato-Ungual-Larimal-Tooth syndrome (ADULT), and Split Hand Foot Malformation (SHFM). The majority of the p63 mutations in EEC-like syndromes involve residues present DNA Binding Domain (DBD), the SAM domain (SAM) and the Transactivation Inhibitory Domain (TID domain). Mutations in the DBD abolish p63 DNA binding ability, while mutations in the SAM domain are supposed to abolish protein-protein interaction while mutations in the TID domain are predicted to impair the suppressive effect of the TID toward the TA domain thus increasing the transactivation activity.

Here, we have performed a systematic study of the transcriptional activity of different p63 mutants using different promoters: skin-specific (K14, IKKalpha, BPAG, EVPL), apoptotic (BAX) and cell cycle (p21).

In addition, we have evaluated the ability of these mutants to induce cell cycle arrest and apoptosis.

From the results obtained, we conclude that: i) there is a clear difference in the transcriptional activity of SAM and TID p63 mutations on skin-specific and apoptosis-related promoters; ii) the specific activity of TAp63alpha and DeltaNp63alpha on skin-specific promoters depends on an intact SAM domain; iii) the TID mutations mostly increase the activity of p63alpha on epidermal promoters.

In addition, while TAp63alpha, and its active mutants, play a role in regulating cell cycle and in inducing apoptosis, DeltaNp63alpha does not affect apoptosis and cell cycle.

EXPERIMENTAL CELL THERAPY IN OSTEOPETROSIS

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Osteopetrosis is a genetic disease characterised by defective osteoclasts. The Autosomal Recessive (ARO) malignant form is fatal, leading to death within the first 3-4 years of life.

Haematopoietic Stem Cell Transplantation (HSCT) cures <50% of cases but often leaves severe neurological damages and other dysfunctions. HSCT may provide the bone with functional osteoclasts, but their appearance is a slow process, during which disease progression continues.

We hypothesise that such outcome could be prevented if readily available committed osteoclast precursors were injected. We optimised the method to obtain cells suitable for therapy and preliminarily tested engraftment and phenotype improvement in animal models. We established a procedure to obtain the best yields of osteoclasts from adult PBMCs exposing the cells for 7 days to 50 ng/ml SCF, 20ng/ml IL-3 and 20 ng/ml IL-6, and for further 7 days with 20 ng/ml GM-CSF. Cells were then cultured for 21 days in the presence of 25 ng/ml M-CSF and 30 ng/ml RANKL.

This procedure was applied to PBMCs, CD14+ and CD34+ cells cultured in regular DMEM or in Invitrogen StemPro34 medium, specifically formulated for the survival and expansion of the HSC population. Best osteoclast yield and performance was obtained culturing PBMCs or CD34+ cells in StemPro34 medium, which resulted in a higher number of osteoclasts, which were also larger and resorbed bone more efficiently than osteoclasts obtained in regular DMEM. These cells could be cryopreserved at -80°C in a medium with 90% FBS and 10% DMSO retaining the ability to differentiate into osteoclasts with a yield indistinguishable from that of freshly isolated cells. Similar positive results were obtained with mouse cell culture prepared as described above.

These cells were injected *in vivo* in animal models after 1-day of treatment with M-CSF and RANKL, testing different cell injection protocols, also in association with untreated CD117+ cells. Injected cells showed the ability to form multinucleated osteoclasts and to improve to some extent the osteopetrotic phenotype of oc/oc mice. In the best working protocol, animals presented with longer survival (1.48 fold increase, p=0.0035), amelioration of ponderal and longitudinal growth (total body, 1.17 fold increase, p=0.012) and tibial length (1.24 fold increase, p=0.012), higher rate of tooth eruption (48% of treated mice), 18% reduction of bone volume, reduced fibrosis and improved haematopoiesis compared to sham-treated mice.

These results provide first hand information on the feasibility of an osteoclast precursor therapy in osteopetrosis.

GENETIC AND CLINICAL ASPECTS OF RARE LYMPHOMAS

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We are investigating of genetic alterations and aspect of quality of life in orphan human lymphomas such as cutaneous lymphomas and those arising in patients with type II mixed cryoglobulinemia associated with chronic HCV infection.

One specific objective is to identify chromosomal areas and genes that can be related to these lymphomas. 20-Primary Cutaneous B-Cell Lymphomas (PCBCL) were investigated for the presence of mutations in the CDKN2A locus coding for $p16^{INK4A}$ and $p14^{ARF}$.

The screening shows 4 samples with altered DHPLC profiles: one PCLBCL-leg type displays aberration of exons 2 and 3, three PCFCL samples showing alterations of either exons 2 and 3 (sample n.11) or only exon 3 (samples 9, 18).

Sample 18 displays a PCR product corresponding to exon 1β of a larger size, suggesting that a possible aberrant rearrangement has occurred. We also investigated the methylation status of $p16^{INK4A}$ and $p14^{ARF}$ promoters through the methylation specific PCR (MSP) technique. Results identified one strong, and possibly three weak methylated cases corresponding to PCFCLs.

We also identified two distinct genetic events in HCV-driven lymphomagenesis. Gain of 3q was found to hallmark low-grade NHL or non-malignant B-cell clonal expansion, as it was observed in 4/6 cases of low-grade Splenic Marginal Zone Lymphoma (SMZL) as well as in the non-malignant lymphoproliferative phase of type II mixed cryoglobulinemia.

Regression of lymphoma after eradication of HCV with antiviral therapy was observed in 3/3 cases of SMZL. Conversely, deletion at 2q22.3 was detected in 4/5 (80%) cases of clinically aggressive Large B-Cell Lymphomas (LBCL).

No response to antiviral treatment was observed in this subgroup. An investigation of Health-Related Quality of Life (HRQoL), has also been performed.

The study population consisted of patients with cutaneous T-cell (CTCL) or B-cell lymphoma, consecutively recruited at IDI-IRCCS.

Data were collected using a dermatology-specific questionnaire, the Skindex-29 (symptoms, emotions, and functioning scales), and an oncology-specific questionnaire, the EORTC QLQ-C30 (15 scales, concerning physical and emotional aspects).

On 95 patients, there were 24 patients with CBCL, 59 with CTCL, and 12 with Sézary syndrome. The most frequent problems appearing from the EORTC QLQ-C30 analysis

were fatigue, pain, and insomnia. The differences among CL types were particularly high in the global health status and emotional functioning scales, with a worse HRQoL in patients with SS, followed by MF, and CBCL. HRQoL impairment in all CL types was higher in women than in men, in patients with probable anxiety or depression, and during worsening of the disease.

DEVELOPMENT OF NEW DIAGNOSTIC APPROACHES FOR TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

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Transmissible Spongiform Encephalopathies (TSEs) are fatal neurodegenerative conditions of humans and animals. The majority of human TSE cases (affecting 1-2 people per million every year) has an unknown aetiology and manifests as sporadic Creutzfeldt-Jakob Disease (sCJD).

The amyloid protein PrP^{TSE}, which derives from the cellular precursor PrP^{C}, is the only available marker of TSEs. Post-mortem detection and biochemical characterization of PrP^{TSE} accumulated into the CNS allow definite diagnosis and classification of TSE diseases. PrP^{TSE} is undetectable in body fluids and this hampers ante-mortem identification of infected individuals and the design of targeted strategies for prevention, control, and treatment of TSEs.

The objective of this project is to improve the diagnosis and the prognosis of human TSEs through the development of new biochemical tests and the optimization of available diagnostic tools.

The first part of the project is the search for disease-specific proteomic patterns. To reach this goal we developed a specific protocol for the collection of body fluids from TSE patients, and from neurological and non-neurological patients. Mass spectrometry and 2D electrophoresis showed that cerebrospinal fluids of sCJD contain γ, ε, and ζ isoforms of 14-3-3 proteins, and that this pattern is not found in inflammatory and vascular disorders of the CNS.

The second part of the project regards the application of the Protein Misfolding Cyclic Amplification (PMCA) to peripheral biological human fluids. PMCA amplifies minute amounts of PrP^{TSE} (using normal brain containing PrP^{C} as a substrate), and has been optimized for reproducibility, specificity and sensitivity on brain samples from an experimental TSE model (263K scrapie in hamsters). We have also adapted the conditions of amplification to human brain and obtained a 10^5-fold of PrP^{TSE} amplification. To overcome the limited availability of non-CJD human brains as a substrate, we are now developing PMCA using brain from humanized mice (Tg Hu-PrP) and the preliminary results demonstrate a high efficiency of conversion.

The last part of the project centers on the improvement of the classification of sCJD subtypes through western blot analysis of PrP^{TSE} types in a series of 225 subjects. In about 30% of these cases we observed the occurrence of PrP^{TSE} types 1 and 2 (as shown by

different electrophoretic migration) with a significant association between disease phenotype and relative abundance of each type.

Although preliminary, the results obtained so far already allowed an updated classification of sCJD variants and may contribute to formulate an early differential diagnosis with other dementias.

DYSREGULATED RAS SIGNALING IN NOONAN SYNDROME AND RELATED DISORDERS: DISEASE GENE DISCOVERY AND FUNCTIONAL STUDIES

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Noonan Syndrome (NS) and the clinically related LEOPARD Syndrome (LS) are genetically heterogeneous Mendelian traits characterized by short stature, facial dysmorphisms, cardiac defects, and variable skin and skeletal anomalies. Increased RAS-MAPK signal traffic due to heterozygous PTPN11 and KRAS mutations cause 50% of NS, while a bunch of amino acid changes impairing PTPN11/SHP2's catalytic activity account for 90% of LS.

Major goal of this project was to identify novel NS/LS disease genes. By using a candidacy approach focused on genes coding transducers with role relevant to RAS signalling, we discovered SOS1, RAF1 and BRAF as novel genes implicated in NS/LS pathogenesis. SOS1 encodes a RAS-specific GEF. SOS1 mutations account for 10% of NS, cluster at residues implicated in the maintenance of its autoinhibited conformation, and promote enhanced RAS-MAPK activation. The phenotype associated with SOS1 defects is distinctive, with a high prevalence of ectodermal abnormalities but normal cognitive development and growth. RAF1 and BRAF mutations were identified in a small percentage of NS and LS. These genes encode serine/threonine protein kinases functioning as RAS effectors. Most RAF1 mutations altered a motif that is critical for protein autoinhibition through 14-3-3 binding, and promoted enhanced ERK activation. RAF1 mutations in two hotspots were strongly associated with hypertrophic cardiomyopathy. BRAF mutations mapped to multiple protein domains, and largely did not overlap with cardiofaciocutaneous syndrome-causing or cancer-associated defects. Selected BRAF mutations promoted variable gain of function of the kinase, but appeared less activating compared than the oncogenic V600E protein.

A second major goal of this project was to characterize functionally a panel of NS/LS-causing PTPN11 mutations. Specifically, we investigated the mechanisms underlying the invariant occurrence of the T42A, E139D and I282V substitutions in NS, and the Y279C and T468M changes in LS. We demonstrated that the Ile-to-Val change at codon 282 is the only substitution at that position perturbing the stability of PTPN11/SHP2's closed conformation without impairing catalysis, while the Thr-to-Ala change, but not other substitutions of codon 42, promotes increased phosphopeptide binding affinity.

The recognition specificity of the C-SH2 domain bearing the E139D substitution differed substantially from its WT counterpart acquiring binding properties similar to those observed for the N-SH2 domain, revealing a novel mechanism of SHP2's functional dysregulation. Finally, we identified the deamination of the methylated cytosine at nucleotide 1403 as the driving factor leading to the high prevalence of the T468M change in LS.

INHERITED EPIDERMOLYSIS BULLOSA: MOLECULAR FINDINGS, DIAGNOSTIC GUIDELINES AND QUALITY OF LIFE EVALUATION

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Inherited Epidermolysis Bullosa (EB) is a clinically and genetically heterogeneous group of rare blistering disorders due to skin and mucous membrane fragility. A recently revised classification distinguishes four major EB types based on the level of blister formation within the skin: EB Simplex (EBS), Junctional EB (JEB), Dystrophic EB (DEB) and Kindler Syndrome (KS). We focused on: i) lethal JEB subtypes [Herlitz JEB (HJEB) and JEB with Pyloric Atresia (JEB-PA)] resulting from recessive null mutations in the laminin-332 genes (LAMA3, LAMB3, LAMC2) or alpha6beta4 integrin genes (ITGB4 and ITGA6); ii) KS, a complex phenotype due to recessive loss-of-function mutations in the kindlin-1 gene (KIND1); iii) dominant and recessive DEB variants caused by mutations in COL7A1 coding for type VII collagen.

Our findings concern: i) the spectrum of unique and recurrent mutations in the Italian population in JEB, DEB and KS; ii) population carrier risk in HJEB; iii) an unusual mechanism of pathogenesis (uniparental isodisomy) in HJEB; iv) sibling-to-sibling phenotypic variations in recessive DEB. We also developed and validated protocols for postnatal mutational screening of JEB and KS as well as a novel approach for the prenatal diagnosis in kindred at risk for EB-PA, which is caused by null mutations in either the alpha6beta4 integrin genes or the plectin gene. Based on the original observation that chorionic villi express these proteins, chorionic villi immunofluorescence examination was shown to represent a novel tool for prenatal diagnosis of EB-PA in kindred carrying unidentified genetic mutations.

In parallel, 180 EB families retrieved from our database, accepted, following an informed consent, to participate to a study aimed at evaluating their Quality of Life (QoL). Self-administered questionnaires (SF-36, Skindex-29, GHQ-12, DLQI, EQD5) adapted for either adults or children were delivered to participants. Preliminary results show a high acceptance rate with 118 participants having sent back the questionnaires. Interim statistical analyses indicate that both adults and children experience a serious impairment of QoL. Patient responses confirm the presence of restriction in different domains of QoL, *i.e.* physical, emotional, and social. Compared to other skin conditions, patients seem to

experience greater difficulties, especially for the physical components. Concerning the family burden of disease the mean values observed are not as high as expected.

Finally, a multidisciplinary, multispecialty task force of experts was convened under the coordination of the National Centre Rare Diseases of the National Health Institute to develop and validate national guidelines for the diagnosis of EB.

DEVELOPMENT OF AN EPIDEMIOLOGICAL AND MOLECULAR INTEGRATED APPROACH FOR THE PREVENTION OF CONGENITAL HYPOTHYROIDISM: PRELIMINARY RESULTS

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Congenital Hypothyroidism (CH) is a rare disease with a prevalence of 3.5 cases/10,000 citizens in Italy. It represents the most frequent endocrinopathy in infancy and the most common cause of preventable mental retardation. The availability of effective screening procedures, the efficient network composed of the 26 Screening Centres for CH active in Italy and the surveillance of the disease performed by the Italian National Register of Infants with CH, have allowed an efficient "secondary prevention" for CH. This represents one of the most important results in the field of preventive medicine in our country and can be a model of intervention for other rare diseases.

In the first year of activity of the project we focused our efforts on the following aims: 1) to use the almost 30-year Italian experience of screening for CH to evaluate the possible impact of "extended newborn screening program" in the Liguria Region; 2) to investigate possible spatial variations in CH risk (spatial clusters) in our country; 3) to study the molecular basis of CH with eutopic gland and thyroid disgenesis.

Some important preliminary results have been obtained. Specifically, data of the extended newborn screening program collected in Liguria Region between June 2005 and May 2008 were analyzed. Six positive cases were identified (argininosuccinate lyase deficiency 1, organic acidemia 2, fatty acid oxidation disorders 3) and an incidence of 1: 6098 live borns was estimated in this Region. As regards the spatial analysis of CH incidence, it was conducted per municipalities on the data collected by the Italian National Registry of Infants with CH between 1995 and 2003.

The presence of spatial clusters with high CH incidence ($\geq$1:1000 live borns) were found spread all over the country suggesting the important role of environmental risk factors in the etiology of CH. Studies aimed at characterizing the molecular basis of CH were performed on children with CH and eutopic thyroid and in a polygenic mouse model

for CH with thyroid dysgenesis. As concerns CH with eutopic thyroid, 21 unrelated patients with this form of CH and a Partial Iodide Organification Defect (PIOD) were studied.

In these patients genes coding for Dual Oxidases (DUOX1 and DUOX2) and DUOX maturation factors (DUOXA1 and DUOXA2) were screened and for the first time a DUOXA2 defect in a Chinese female with CH, PIOD and mild permanent hypothyroidism during childhood was found. Moreover, the analysis of the coding-region of DUOX2 gene in further 10 CH children with eutopic thyroid, identified 6 new mutations involving exon 22, exon 17, exon 23, exon 24, and exon 21.

Finally, for what concerns molecular basis of thyroid disgenesis, 143 DHTP/bc mice were genotyped using 235 Single Nucleotide Polymorphisms (SNPs).

Analysis of genotyping data revealed two chromosomal regions associated to CH: one on Chr 2 (with a LOD score of 11.2) and another on Chr 5 (with a LOD score of 2.5). 800 genes have been mapped on region of Chr 2 associated to the CH phenotype; about 400 of these are expressed in the thyroid. By using SNPs analysis 2 new candidate genes have been identified: calpain 3 and Dnajc17.

CHARACTERIZATION OF HIPK2 THAT BY ASSOCIATING WITH MECP2 MIGHT FUNCTION AS A MODIFIER GENE IN RETT SYNDROME

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Mutations in the Methyl CpG-binding Protein 2 (MeCP2) gene, located on Xq28, are responsible for almost all cases of classic RTT. Conversely, less than half of the patients with one of the variant forms of RTT carry mutations in MeCP2.

It seems, thus, that other genes are involved in causing RTT; moreover the fact that there are patients with milder phenotypes in spite of severe mutations argues that modifier genes might restrict the clinical outcome by regulating MeCP2 functions.

To search for MeCP2 interacting proteins possibly involved in RTT we performed a yeast two-hybrid screening and identified among the positive clones HIPK2 (Homeodomain Interacting Protein Kinase 2) that belongs to a family of Ser/Thr kinases originally identified as corepressors for homeodomain transcription factors.

HIPK2 has a clear role in regulating cell growth and genotoxic stress-induced apoptosis.

Furthermore, its involvement in the nervous system is indicated by the neuronal defects of null mice that partially overlap those observed in MeCP2 ko mice.

Since important MeCP2 functions in the nervous system are regulated by its phosphorylation we found it interesting to analyze the functional role of its interaction with HIPK2.

We have thus confirmed that the two proteins associate *in vitro* and *in vivo* and phosphorylation assays have shown that MeCP2 is significantly phosphorylated by HIPK2 *in vitro*. Importantly, these assays have also allowed us to establish that Ser80 within the MBD of MeCP2 is a specific target of HIPK2.

Functional assays have shown that ectopic MeCP2 causes an increase in cell death and an additive effect of the two proteins in inducing apoptosis in cultured cells was observed. Importantly, the role of MeCP2 in inducing apoptosis together with HIPK2 is lost when Ser80 is mutated or a kinase dead derivative of HIPK2 is used.

Presently we are analyzing whether MeCP2 is a target of the kinase also *in vivo* and the role of the interaction for the nervous system.

In favor of the hypothesis that the two proteins work in a common molecular pathway we have shown by immunohistochemistry experiments that the expression

pattern of the two proteins in the brain of adult mice is highly similar. We therefore believe that these studies are relevant for understanding whether this novel MeCP2 interactor acts as a modifier gene influencing disease severity in RTT patients with mutations in MeCP2.

microRNA EXPRESSION PROFILE OF PARATHYROID CARCINOMAS

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The pathogenesis of parathyroid cancer remains unclear. Recently, the loss of the oncosuppressor HPRT2 gene product, parafibromin, has been demonstrated to be involved in the Hyperparathyroidism-Jaw Tumor (HPT-JT) syndrome and in a consistent set of sporadic parathyroid carcinomas. This finding highlighted the role of the Wnt/beta-catenin pathway in parathyroid carcinogenesis. Nonetheless, detailed understanding of parathyroid oncogenesis would facilitate addressing scientific and clinical challenging. MicroRNAs (miRNAs) are a new class of small, noncoding RNAs implicated in development and cancer. A deregulated miRNA can orchestrate the aberrant expression of several target genes.

The aim of the present study was to identify deregulated miRNAs in parathyroid cancers compared to normal parathyroid tissues. We performed a low-density array-based profiling of 4 parathyroid cancers and two normal parathyroid biopsies. All carcinomas showed a mutation in HPRT2 gene and were negative for parafibromin immunostaining. Out of 362 human miRNAs assayed, 279 (77%) were expressed above background levels in all samples. Hierarchical clustering based on the expression of these miRNAs correctly classified the normal specimens from the tumors. For all expressed miRNAs, we calculated Fold Change ratios (FC) between normal and tumoral parathyroid specimens on median normalized RQs. As threshold for significant different expression, we set FC=5 or 0.2, for over or down-expression, respectively.

At the indicated cut-off, 15 miRNAs were significantly down-expressed, while just 3 miRNAs displayed a statistically significant overexpression in all parathyroid cancer specimens. SAM analysis setting the minimum fold change for log-transformed data at 2.5 and maximum q-value at 49.1%, identified hsa-miR-296 and 139 being negatively related with tumor presence and hsa-miR-503 and 222 being up-regulated in parathyroid carcinomas. In particular, miR-296 was able to discriminate between parathyroid cancers and normal glands (p=0.0012) with a null misclassification rate at the cross-validation procedure. To further investigate the expression of hsa-miRNA-296, we analyzed its expression profile in 13 parathyroid sporadic adenomas, 4 atypical adenomas and 2 metastasis. Hsa-miRNA-296 expression levels were definitely low in parathyroid cancers and metastasis as well as in atypical adenomas, while in sporadic adenomas they were reduced but not significantly different from normal samples. In conclusion, we identified a

set of 4 miRNAs differently expressed in parathyroid carcinomas versus normal parathyroids. In particular, the down-regulated has-miRNA-296 was shown to distinguish unequivocally between parathyroid carcinomas and adenomas. Further studies are needed to define the target genes of has-miRNA-296 in parathyroid tissue and its regulation.

P. ORPHAN DRUGS: GENERAL DEFINITION AND RELEVANCE FOR THE DERMATOLOGIST

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Orphan drugs are defined as those products not distributed by the pharmaceutical industry because they are not commercially viable even if they meet a general public health need.

However, a substance used in the treatment of a frequent disease may also have an as yet undeveloped use as orphan product.

In real terms, orphan drugs fall under the following categories: i) products aimed at the treatment of rare diseases; ii) products taken off the market for commercial or therapeutic reasons; iii) products that have not been developed. At present, the issue of orphan drugs is under consideration in the case of rare diseases.

In Europe, a rare disease is one which affects no more than 5 on 10,000 individuals.

The WHO has so far classified 5,000 rare pathologies for the most part caused by genetic anomalies. In these pathologies, diagnosis is the first problem that has to be faced but a second problem is what approach to adopt in terms of therapy. One of the greatest obstacles in ensuring suitable treatment for these patients is the pharmaceutical industry's unwillingness to develop drugs at normal marketing conditions because, in the absence of incentives, the costs of the process would not be recovered from sales.

The development of drugs for rare diseases has been concentrated in certain areas (particularly cancer and metabolic diseases) whereas no or few drugs have been approved for other areas including those of interest for dermatologist. This is unfortunate because rare diseases are present in dermatology and so this issue is of importance to the practitioner.

GENOTYPE-PHENOTYPE CORRELATIONS IN THE CMT NEUROPATHIES: DEFINITION OF A CLINICAL AND GENETIC DIAGNOSTIC FLOW-CHART

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Charcot-Marie-Tooth (CMT) neuropathies are severe forms of disability characterized by progressive impairment of motricity and sensibility that affects quality and duration of life. On the basis of electrophysiology and histopathology, CMT has been divided into primary demyelinating, CMT1, and primary axonal, CMT2, neuropathies. CMTs are a very heterogeneous group of disorders, which have been associated to at least 25 genes. It is mostly impossible to predict the type of locus/gene associated with a specific CMT on the basis of the phenotype with few exceptions.

Only few Medical Institutes can provide a broad range of molecular screening and patients often need to visit several Institutes to expand or complete the genetic screening. The request for a definite molecular diagnosis of CMT has relevant consequences. First, although a specific therapeutic approach is not yet available, clinical trials for specific forms of CMT are ongoing, such as ascorbic acid or progesterone for CMT1A. Therefore, the identification of the molecular defect may provide the access to future treatments that may be specific for some CMT instead of others. Second, the molecular definition of CMT disease can avoid continuous repetition of exams and hospitalization, which have an evident social and economic impact. Finally, the definition of CMT disease can provide information about the clinical outcome of the disease and the inheritance risk.

At San Raffaele Hospital, we organized a specialized team, where clinicians, geneticists and scientists are strictly collaborating to evaluate the different aspects of CMT neuropathies. Our cohort now includes 180 patients, all carefully evaluated from the clinical, electrophysiological and, histopathological point of view, in order to better define clinical diagnosis and orient molecular testing. We have organized a systematic genetic screening of 15 most frequently mutated CMT genes.

To date, 89 probands have now concluded the molecular screening process. Among them, 47% are affected by demyelinating, 29% by axonal and 24% by intermediate forms of CMT. In this cohort, both sporadic (62%) and familial (38%) cases are represented. Molecular analysis led to the identification of mutations in 36/89 cases (40%), which rises to 65% for patients with positive family history. We could detect a genetic alteration in

52% of demyelinating cases (79% with positive family history), 38% of axonal (71% in familial cases) and 19% in intermediate CMTs (17% in familial cases). We also identified five new mutations in the following genes: MPZ (D224Y), EGR2 (D383H), MFN2 (A738V), GDAP1 (P59fsX61), HSP27 (S135C).

IDENTIFICATION OF miRNA/TARGET GENE PAIRS INVOLVED IN HEREDITARY BREAST CANCER

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We performed a transcriptome analysis of coding and non coding RNAs (miRNA) from a group of BRCA1, BRCA2, BRCAX (familial cases with no mutations in BRCA1 or BRCA2 genes) and sporadic breast cancers using the microarray technology for identifying possible miRNA dependent mechanisms involved in this we tumor type.

We identified 106 genes ($P<0.005$) and 100 miRNAs ($P<0.05$) that were differentially expressed between the groups of familial and sporadic breast cancers included in our study. In particular gene expression analysis confirmed the strong difference between BRCA1 group and the other cases, while miRNA analysis highlighted the prevalent expression of these molecules in the sporadic cases. Cluster analysis of the samples showed that the BRCAX group consisted of two sub-groups, one similar to sporadic tumors, the other with characteristics similar to the group of patients with BRCA1 mutations. These results were observed both for coding and non coding transcripts, thus supporting the genuine existence of the two sub-groups.

To identify miRNA-target gene pairs deregulated in breast cancer we integrated the two analyses. First we searched the public databases for the putative miRNA regulators (predicted to bind complementary sequences) of the genes we found differentially expressed among our breast cancer groups. We then confirmed their presence in the miRNA lists we generated from our expression profiling. We identified 24 miRNA inversely correlated with 76 genes. We focused on one pair differentially expressed between Estrogen Receptor (ER)-positive and ER-negative cases and involving a gene whose expression is known to be inversely correlated to that of ER.

This gene has also been proposed as a negative regulator of BRCA1 and its possible regulatory miRNA was highly expressed in the BRCAX sub-group that exhibited the characteristics of BRCA1-mutant cases. QRT-PCR analysis on a panel of breast cancer cell lines and on 293T cells confirmed the inverse expression of this putative pair. We functionally validated the interaction observed by cloning the 3' UTR sequence of the gene containing the predicted miRNA binding site in a luciferase reporter construct in 293T cell line. We are testing the role of this miRNA in breast cancer by transfecting it in breast cancer cell lines and analyzing changes in expression of the endogenous target gene, as well as of BRCA1 and ER.

INNOVATIVE BURKITT'S LYMPHOMA THERAPY

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Burkitt's Lymphoma (BL), one of the most aggressive human cancers, is a very rare malignancy in the Western world (sporadic form), while is frequent among children from areas like Central Africa (endemic form). Systemic chemotherapy, the present treatment of choice for BL at all stages, has high toxicity and has an overall survival rate correlated to the stage of the disease at diagnosis and concomitant pathologies (*e.g.* HIV-seropositivity).

Most BL are characterized by t(8;14, 2 or 22) chromosomal translocations juxtaposing the c-myc oncogene to the Ig *loci*.

Consequently c-myc becomes up regulated by the now proximal Eμ enhancer, at the 5'of the Ig *locus*, promoting cell hyper proliferation. We previously demonstrated that, in BL cells with the t(8;14) translocation, a Peptide Nucleic Acid (PNA) complementary to the intronic Eμ sequence (PNAEμ) specifically inhibited expression of the translocated c-myc and caused *in vitro* cell growth arrest.

We recently completed a series of test required to enter PNAEμ in phase I/II in patients, like -definition of pharmacokinetics (including the persistence of the therapeutically active portion of PNAEμ) and lack -of toxicity in an *in vivo* model system, -of immunogenicity in immunocompetent mice, -of mutagenicity or clastogenicity (as detectable by standard assays).

We initially studied a BL model of SCID mice inoculated *s.c.* with BRG-BL cells.

This model presented the advantage of developing easily detectable and measurable tumors. We demonstrated that chronic administration of PNAEμ to these mice, already inoculated with BL cells, caused: increased latency of tumor appearance, relevant decrease of final tumor size and necrosis.

However the efficacy of PNAEμ needed to be tested in a model more similar to the human adult sporadic form of BL. BRG-BL cells were transfected with the firefly luciferase gene and inoculated *i.v.* into SCID mice giving origin to disseminated lymphomas. BL cells growth was detected in mice upon *i.p.* injection with D-Luciferin with an IVIS Xenogen imaging system. Bioluminescent signals from BRG-BL-Luc cells, as captured by the camera system, were recorded, integrated, digitalized, displayed, and quantified (in photons/second) using the Living Image program.

With this type of analysis we determined that treatment with PNAEμ specifically caused significant inhibition of BL cell expansion as confirmed by inspection at necropsy. In particular a reduced invasion was apparent at the main sites of BL cell growth (similar to the human BL): peritoneal fat, brain and rachis. Altogether, the data support the potential therapeutic value of PNAEμ in Burkitt's Lymphoma.

NOVEL EXPERIMENTAL APPROACHES FOR INVESTIGATION ON NEW THERAPIES AGAINST RARE HUMAN BONE TUMORS

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Human bone malignancies including Osteosarcoma (OS) and Ewing's Sarcoma (ES), are rare tumors in both adults and children, characterized by a high biologic aggressiveness.

The strategy of treatment is based on a combined modality of surgery and chemotherapy. Chemotherapy (a combination of 4 drugs), is delivered before and after surgical removal of the tumor (neoadjuvant chemotherapy).

However, the most recent improvements in the cure rate of patients with localized disease have been achieved by dose-intensification, in turn paying the price of acute severe toxicity and secondary malignancies. Moreover, 35% of patients show tumor resistance to systemic therapies and treatments for high-risk patients are still completely inadequate and no new drugs have reported to be really active and useful in sarcomas.

Thus, innovative treatment modalities are indeed very welcome and needed. In this project we proposed some *in vitro* and *in vivo* models, to evaluate potential new therapeutic strategies aimed at inhibiting the growth and metastatic behaviour of human bone tumors. The first issue of the project was to investigate the significance of ezrin in bone malignancies.

Moreover, we investigated expression and function of V-ATPases in osteosarcoma and Ewing's sarcoma cell lines. We also performed experiments aimed at analyzing molecular composition of microvesicles released from bone-marrow malignancy cells. Virtually conclusive data have shown that ezrin is involved in many activities of bone marrow malignancies, including multidrug resistance and microvesicle release. The data definitively show that ezrin is instrumental for a full function of Pgp-1 in Osteosarcoma (OS) MDR cells. In fact, ezrin was linked to Pgp-1 exclusively in MDR cells, as compared to parental sensible cells. In turn this linkage is related to the membrane localization of Pgp-1.

Through the use of a deletion mutant of ezrin we transfected the MDR OS cells, showing that overexpression of a small inefficient ezrin leads to loss of function of MDR cells, that become sensible to chemotherapeutics and loose Pgp-1 membrane expression. As far as Pgp-1 is concerned we found this protein expressed on MDR OS released microvesicles.

Moreover, also V-ATPases have shown to participate to the malignant behaviour of rare bone tumors and some V-ATPases inhibitors have proven to interfere with the metastatic potential of these tumors. MRI-guided MRS approaches have shown that bone marrow malignancies are clearly acidic and that inhibition of V-ATPases activity, through Proton Pump Inhibitors (PPI) increase the pH of these tumors. Consistent with PPI effect on tumor acidity we also found both increased sensibility of OS tumors to *in vivo* treatment with PPI in terms of both chemosensibilization and a direct antineoplastic effect. Lastly, treatment of an extensive panel of osteosarcoma and Ewing's sarcoma cell lines bone-tumors derived

cells with small molecule inhibitors against IGF-IR or c-src, recently identified as specific tyrosin kinase inhibitors, have shown to be effective in inhibiting the tumor cell growth.

Altogether these preliminary results suggest that ezrin and proton pumps may represent newly-identified molecular targets for new therapeutic strategies against rare bone malignancies.

GENOTYPE/PHENOTYPE ANALYSIS OF NEURODEGENERATIVE AND AGING-PRONE SYNDROMES CAUSED BY MUTATIONS IN THE DNA DAMAGE RESPONSE/REPAIR PATHWAY

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Inherited syndromes caused by alterations of DNA repair genes frequently associate with early-onset progressive neurodegeneration, premature ageing and other major extra-neurological features.

These diseases include Ataxia-Telangiectasia (A-T), Ataxia-Telangiectasia-Like Disease (ATLD) and Nijmegen Breakage Syndrome (NBS), caused respectively by mutations in ATM, Mre11 and NBS1 genes involved in DSBs repair; Werner Syndrome (WS) mutated in WRN gene involved in DNA replication, recombination repair, transcription and incorrect handling of stalled forks with accumulation of DSBs; Ataxia Oculomotor Apraxia type 1 (AOA1) and type 2 (AOA2) caused by the genes APTX and SETX involved in SSBs repair. This project was aimed to elucidate the role of these genes in determining specific phenotypes.

As for WRN, we found that cells from WS individuals to have a striking accumulation of DSBs after replication-perturbing treatments, which was prevented by knock-down of the MUS81 endonuclease, unveiling a back-up pathway that ensure survival after DNA damage in WS cells.

Furthermore, we evidenced that stalled replication forks collapse in the absence of WRN, as shown by PCNA removal from chromatin and loss of viability of ongoing forks upon DNA replication arrest. Finally, we found that MUS81 knock-down in WS cells results in reduced chromatin recruitment of recombination enzymes, decreased yield of SCEs and reduced survival after replication arrest.

Thus, we provide new evidence highlighting the requirement of WRN to avoid accumulation of DSBs and fork collapse after replication perturbation and that prompt MUS81-dependent generation of DSBs is instrumental for recovery from HU-mediated replication arrest under such pathological condition. Regarding SETX, a protein of around 350,000 KDa, we have successfully expressed it as a GFP-fusion in a variety of cell lines and found that it produces dramatic effects on cell cycle progression.

This finding, which is currently being investigated in detail, also provides an explanation as to why we were unable to generate cells stably expressing SETX.

We have produced a SETX-specific high affinity rabbit antibody used for western blot to monitor the levels of SETX proteins for diagnostic purposes, and that we are currently

employing to immunoprecipitate endogenous SETX complexes for mass spectrometry analysis and identification of SETX-interacting proteins.

As for A-T, in a collaborative study, we have set up an immunofluorescence analysis ATM phosphorylation that coupled with flow cytometry allows quick diagnose of A-T patients and A-T heterozygotes directly from resting peripheral blood lymphocytes.

INNOVATIVE MANAGEMENT OF PATIENTS WITH DIFFUSE MALIGNANT PERITONEAL MESOTHELIOMA: CLINICAL-DIAGNOSTIC PATHWAY AND NEW THERAPEUTIC TARGETS. PRELIMINARY RESULTS

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Background. Diffuse Malignant Peritoneal Mesothelioma (DMPM) is a rare and rapidly lethal neoplasm. In recent years, the combination of cytoreductive surgery and Hyperthermic Intraperitoneal Chemotherapy (CRS+HIPEC) has resulted in a significant survival improvement, as compared to historical controls. Little is known about DMPM genetic and molecular features. In the present study, we assessed new prognostic indicators and therapeutic targets in a large series of DMPM undergoing CRS+HIPEC.

Methods. From a prospective data-base of 86 cases, we selected 66 DMPM. Cases with well-differentiated histology or second malignancies were excluded. All the patients underwent peritonectomy procedures and closed-abdomen HIPEC with cisplatin and doxorubicin. The prognostic significance of age, sex, carcinomatosis extension, Completeness of Cytoreduction (CC) and HIPEC drug schedule was tested by multivariate analysis. We evaluated the expression of members of the Inhibitor of Apoptosis Protein (IAP) family (survivin, IAP-1, IAP-2, X-IAP) and the presence of telomere maintenance mechanisms (telomerase activity and Alternative Lengthening of Telomeres [ALT]). In 15 patients, EGFR, PDGFRA and PDGFRB expression and phosphorylation were immunohistochemically and biochemically analysed and automatically sequenced. The cognate ligand expression was investigated by real-time PCR. Additionally, we explored RTK downstream pathways status through mutational and biochemical analysis of PI3KCA gene PTEN/AKT, ERK, mTOR and its effector S6.

Results. Median follow-up, Overall (OS) and Progression-Free Survival (PFS) were 30.5 (range: 1-118), 40 and 17 months. Median. CC independently correlated to OS and age to PFS. IAPs were simultaneously up-regulated in a high percentage of DMPMs (survivin was present in >90% of cases). Survivin gene knockdown in mesothelioma cells resulted in a significant and time-dependent decline of *in vitro* growth and enhanced rate of spontaneous and drug-induced apoptosis. At least one telomere maintenance mechanism was present in 86% of DMPMs. Telomerase activity correlated to poor OS and PFS, whereas ALT failed to significantly affect clinical outcome. Immunohistochemical and western blot analyses showed EGFR, PDGFRA and PDGFRB expression and activation in most cases. Autocrine loop activation of these receptors was suggested in all cases by the expression of the related cognate ligands, in absence of receptor gain of function mutations.

No PI3KCA mutations were found, while all DMPMs showed expression of PTEN and expression/activation of AKT, ERK, mTOR and S6.

Conclusions. CRS+HIPEC is associated to encouraging survival results. Both telomere maintenance mechanisms, telomerase activity and ALT are present in DMPM and differentially affect prognosis. EGFR, PDGFRA and PDGFRB are promising molecular targets for tailored treatments.

P. CHARACTERIZATION OF Wnt/β-CATENIN PATHWAY, IGF-2 METHYLATION STATUS, MIR PROFILES AND CELL SIGNALLING IN HEPATOCARCINOMA AND HEPATOBLASTOMA CELL LINES

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Hepatocellular Carcinoma (HCC) and Hepatoblastoma (HB) are two hepatic cancers characteristic of the adulthood and childhood, respectively. The high mortality associated with these diseases is mainly attributed to the inability to diagnose HCC and HB at early stages.

Several signalling pathways, such as Wnt/β-catenin and Insulin-like Growth Factors (IGFs), have been associated with the pathogenesis and prognosis of several tumours. Wnt/β-catenin signalling pathway controls cell proliferation and body patterning throughout development; IGF-2 gene expression is crucial for normal development, being highly expressed in foetal liver, and its concentration declining after birth. Alterations in the Wnt signalling pathway and/or mutations in β-catenin encoding gene, are involved in a high percentage of human HCC and HB. Furthermore, dysregulation of normal IGF-2 gene promoters usage, also due to DNA methylation status, has been observed in several cancers, including HB.

The aim of this study was to identify new molecular markers for HCC and HB, by investigating the gene, protein and microRNA expression profiles in HCC (Hep3B, HepG2, HLE), and in HB (HUH6) human cell lines, in comparison to human primary hepatocytes. Furthermore, IGF-2 methylation status of its different promoters was examined.

To this end, the expression of 113 genes involved in the Wnt/β-catenin pathway was analysed by microarray. As a common pattern of the hepatic cancerogenesis, a significant modulation in the expression of 8 genes was observed in all tested tumour cell lines analysed, as compared to normal hepatocytes. In particular, 4 genes were up-regulated (FZD7, NLK, RHOU, SOX17) and 4 were down-regulated (TCF7L2, TLE1, SLC9A3R1 e WNT10A), as also confirmed by qRT-PCR.

The microRNA profiles are markedly altered in cancers and some of them have a causal role in tumorigenesis. Among them we focused on the expression of microRNA-21

oncogene, finding that it was overexpressed in the cell lines compared to normal hepatocytes, while the liver-specific microRNA-122 was downregulated. Downregulation of microRNA-122 is associated with hepatocarcinogenesis as described elsewhere. Surprisingly, we found that microRNA-483, located in chromosome 11p15 in one of the IGF2 gene intron, was finely regulated.

To assess the role of IGF2 gene and microRna 483 the methylation status of the four different IGF2 promoters, was analysed by Methylation Specific PCR (MSP): in particular we focused on promoters 2, 3 and 4. Overall promoter-specific levels of IGF2 transcription has been evaluated by qRT-PCR. Dysregulation of pleiotropic anti-apoptotic proteins, growth factors, receptors and their downstream components represent a central pro-tumorigenic principle in human hepatocarcinogenesis. In order to perform a global molecular characterization of the four HCC and HB cell lines, the expression of 224 proteins that covers biological pathways such as apoptosis, cell cycle, and signal transduction was analysed using a protein array system. The protein expression profiles were significantly different among the four cell lines analysed. The commonly modulated ones were 8 proteins: 4 proteins belong to the cell cycle category (c-Abl, Cdc25, Cdk4, and Ciclin D3), 2 to the cytoskeleton category (Cytokeratin 4 and 13), 1 to apoptosis (DAPK pS^{308}), and 1 to signal trasduction (GRB-2). Protein modulation was confirmed by Western blot analysis.

In conclusion, by gene, protein and microRNA expression profiling, several possible molecular markers for HCC and HB have been identified. In particular, the increased GRB-2 expression observed in the cell lines analysed seems to be particularly interesting due to its possible role in the genesis of other types of tumours.

This work is performed within the frame of the project "Tackling rare diseases yet lacking diagnosis and/or prognosis: a pilot project integrating data collection and experimental studies" supported by a NIH-ISS 2007-2009 grant.

AUTOIMMUNE PEMPHIGUS (AP): DYNAMICS OF AUTOREACTIVE B CELLS AND QUALITY OF LIFE EVALUATION

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AP is a rare but life-threatening bullous disease of the skin and mucous membranes. AP is mediated by autoantibodies against keratinocyte cell surface antigens, desmoglein 1 and 3 (Dsg1 and Dsg3). To study the repertoire of autoreactive memory B cells in AP patients at various disease stages (untreated, corticosteroid-treated, in remission) and following rituximab treatment for severe disease, we have applied a previously developed method for the efficient immortalization of human B cells. Several human Monoclonal Antibodies (MAbs) reactive for various epithelial antigens were cloned: 19 anti-Dsg3 and/or Dsg1 MAbs and 16 MAbs recognizing different keratinocyte antigens.

The MAbs have been characterized by: i) ELISAs based on Dsg1 and Dsg3 ectodomain; ii) staining of different epithelial tissues and cell substrates; iii) immunoblotting and immunoprecipitation analysis using keratinocyte extracts.

Seven of the anti-Dsg Mabs were IgG4 and 12 used kappa light chain, 14 stained live HaCaT with an intercellular pattern and 10 stained at least one of the analyzed tissues. Preliminary data indicate periplakin and a ≅100 kDa protein as possible targets of MAbs recognizing epithelial antigens other than Dsg1/3. The pathA recognising Dsg1/3 as well as other epithelial antigens could represent valuable tools for disease diagnosis and for investigating mechanisms of blister formation in AP.

In parallel we have focused on the analysis of AP natural history and of the effects of disease and therapy burden on Quality of Life (QoL) and psychological status. a set of instruments for qol evaluation has been routinely used for all ap cases followed in our institute (220 patients enrolled). Hospitalized patients received the sf-36, skindex-29 and GHQ-12 questionnaires and reported on the perceived severity of disease. In parallel, disease severity was assessed using the Physician Global Assessment (PGA) and the ikeda scoring system. A preliminary analysis of the first 139 patients showed strong impact of ap on health status especially in women, older subjects and patients with mucocutaneous lesions. A significant correlation between disease severity and lower sf-36 values was also noted. AP patients presented a markedly impaired overall QoL compared with healthy controls on all three skindex-29 scales ($p<0.001$). Disease severity was also significantly associated with all three skindex-29 scale scores, for both pga and ikeda values ($p<0.05$). A high percentage of patients presented psychiatric non psychotic symptoms. overall, the introduction in daily clinical activities of QoL evaluation instruments has been helpful, adding more information for clinical reporting and patient management.

P. THE ITALIAN EXTERNAL QUALITY ASSESSMENT (EQA) IN GENETIC TESTS: THE VI EQA SCHEME

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The Italian External Quality Control (EQC) started in 2001 at the Istituto Superiore di Sanità. The following four EQC disease schemes are offered: Cystic Fibrosis (CFTR gene), Beta-Thalassemia (Hbb gene), Fragile-X syndrome (FMR1 gene), the Adenomatous Polyposis Coli (APC gene); the cytogenetics scheme covers prenatal, postnatal and oncological diagnosis.

82 Public laboratories have been enrolled on a voluntary basis, distributed on the National territory. Five trials have been performed and concluded. Results showed that there has been an improvement in the use and the interpretation of molecular genetic tests. The average genotyping error rate observed over the five years was 0.6%, 0.3%, 5% and 4.7% in the Cystic Fibrosis, Beta-Thalassemia, Fragile-X syndrome and Adenomatous Polyposis Coli scheme respectively.

The percentage of complete reports in cytogenetics increased over the period. However, lack of information or inadequacy in reporting are still observed. On the other hand, as has been indicated in other international surveys for quality assessment, it will be only after several years of testing experience and participation in quality assessment schemes that a significant reduction in laboratory errors will be possible.

On the basis of the experience acquired until now and in order to harmonize the activity of our schemes with existing European ones, we have developed a web-based system that has been used for the VI trial. A total of 96 Public laboratories have been enrolled by website through an account, with an increase of about 17% compared to V trial. In particular: i) 30 laboratories out of 96 were registered for cytogenetics; ii) 36 for molecular genetics; iii) 30 for cytogenetics and molecular genetics.

The number of respondents was: 46/49 (94%) for Cystic Fibrosis; 22/23 (96%) for Beta-Thalassemia; 17/21 (81%) for Fragile-X syndrome; 6/6 (100%) for Adenomatous Polyposis Coli; 28/33 (85%) for oncological cytogenetics; 37/43 (86%) for prenatal diagnosis; 48/55 (87.3%) for postnatal diagnosis.

The section of the web-based system restricted to the Steering Committee and assessors is in progress in order to develop a more clear marking system, either in molecular genetics and in cytogenetics, to better identify the performance of laboratories.

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RESEARCH PROJECT "INFANT BOTULISM": THE FIRST TWELVE MONTHS

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In the frame on the bilateral Italy (ISS)-USA (NHI, Office for Rare Diseases) agreement on joint research and development of public health actions on rare diseases, "Infant Botulism" project was developed. Infant Botulism (IB) is an orphan (rare) disease that affect infants aged less than one year. From 1976 to 2007 less than 3,500 cases were worldwide recognized. Despite Italy reported the largest number of cases in Europe, the disease is little known and diagnoses are made mainly by clinicians who have a familiarity with the illness. The main objectives of this project are to improve knowledge of the disease in Italy by training physicians to the suspicious and improving public awareness, standardization of a therapeutic protocol, inclusion of the illness in the Italian National Register of Rare Diseases. The project started on 1st May 2007, is structured in three work packages: WP1-Educational program; WP2-Diagnostic methods; WP3-National Reference Center for Botulism (NRCB), Website on Infant Botulism.

During the first year of work, WP1 developed educational courses to evaluate the medical knowledge of IB. Sixteen Scientific Societies were involved. Written and web-version questionnaire were submitted to physicians. Typical case with a clinical syndrome of IB was described and the specific answers regarding clinical management and differential diagnosis were posed. Preliminary results showed that only 6% of physicians consider IB as first diagnosis. An entire session dedicated to botulism particularly in infants, was also included in "Antidotes in Depth 2008 and Chemical Emergencies Clinical and Public Health Issues", International Continuing Education Course in Clinical Toxicology (Pavia, October 14-18). Medical information will be distributed with two different types of brochures addressed to medical staff, public and parents.

About diagnostic methods (WP2), the use of molecular methodologies were investigated in order to have a rapid diagnosis of the cases. Extraction and purification of DNA from Botulinum Neurotoxin (BoNT) producing *Clostridia* strains were optimized. Multiplex gel-based PCR and SYBR Green Real Time PCR were successful utilized to detect respectively bont/A, bont/B, bont/E, bont/F genes and bont/A gene. In order to develop a Multiplex probe-based Real Time PCR, all bont sequences reported in GeneBank were submitted to *in silico* analysis to detect best primers and probes. Since the genoma of BoNT-producing *Clostridia* containing a low percentage of C/G, MGB and LNA probe were chose.

About WP3, the procedure to obtain BabyBIG in Italy and link between the Infant Botulism Treatment and Prevention Program (IBTPP) and NRCB websites were defined.

MYH9: POSSIBILITIES FOR A NUCLEAR ROLE

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We have identified MYH9 as a target gene of the homeodomain-containing transcription factor Prep1 by screening a promoter microarray with DNA originated from chromatin immunoprecipitations with Prep1 antibodies. Analyses in Prep1 hypomorphic mutant mice show a decrease in the endogenous levels of MYH9, as compared to wt littermates.

In addition to our results showing a nuclear interaction between Prep1 and MYH9, the above data suggest that Prep1 and MYH9 may generate a regulatory loop.

Since the interaction of MYH9 with Prep1 occurs in the nucleus we asked if the former protein contained Nuclear Localization (NLS) and Nuclear Export Signals (NES).

An *in silico* search has identified a number of NLS, but no NES. Furthermore, MYH9 also contains a number of canonical leucine-zipper motifs, suggesting its association with leucine-zipper containing transcription factors or the possibility of forming homodimers.

We present preliminary results on the subcellular localization and interaction with Jun/Fos family transcription factors of MYH9.

MOLECULAR MODELING OF NIPBL MISSENSE MUTATIONS: AN ADJUNCT TOOL FOR THE COMPREHENSION OF GENOTYPE-PHENOTYPE CORRELATIONS

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Cornelia de Lange Syndrome (CdLS) is a rare multisystem disorder characterized by facial dysmorphisms, upper limb abnormalities, growth and cognitive retardation. The main causative gene, NIBPL, is responsible for CdLS in about 50% of patients; the encoded protein, delangin, belongs to the adherin family and is involved in sister chromatid cohesion, DNA repair and long range gene regulation.

Within a cohort of 92 CdLS patients with a phenotype scored as "severe", "moderate" or "mild", we identified 46 NIPBL mutations, including 8 missense mutations.

Genotype-phenotype correlation are not clearly defined for NIPBL mutations, in particular for the, missense ones, which appear to be associated with a variable phenotype. Aiming at establishing whether or not a molecular modeling approach might be in keeping with the clinical presentation of the identified carriers of missense mutations we addressed the modelling of the major part of NIPBL protein, which is yet unavailable.

Indeed the X-ray structure has been deposited in PDB only for the second part of the protein. We built up a 3D model of the human NIPBL protein using the Fold Recognition approach. Several different structural and evolutionary criteria enabled us to choose as model the QBK1 Rod-like C-shape structure as it showed a convenient thermodynamic structure and the best score following validation with VERIFY 3D and energetic minimization by GROMACS program.

Up to day *in silico* mutagenesis of three substitutions was carried on by this model: p.Arg1856Gly associated with a very severe clinical score, p.Arg2298Leu and p.Arg2298Cys both affecting the same residue and underlying a moderate clinical phenotype. Only Arg1856Gly affects the folding region, losing crucial intramolecular interactions and exposure to the solvent, while the other two variants cause mild conformation changes. These preliminary results indicate the usefulness of bioinformatic tools in the comprehension of genotype-phenotype correlation.

Evaluation of other missense mutations mapping within the "modeled" region is in progress.

CHARACTERIZATION OF GENETIC AND CYTOGENETIC ALTERATIONS IN SALIVARY GLAND TUMORS

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Tumor development and progression are multistep processes caused by genetic aberrations and epigenetic modifications which result in the activation or inhibition of biological events as cell proliferation, apoptosis, genomic stability, angiogenesis, invasion and metastasis.

Genetic and epigenetic changes can be analysed by using an increasing number of large-scale genomic technologies and techniques of molecular genetics/cytogenetics.

Salivary Gland Tumours (SGTs) are rare tumors of the neck and head, with an overall incidence in the Western world of approximately 2.5-3/100,000/year.

SGTs are remarkable for their histopathologic and biologic diversity; they include benign and malignant tumors of epithelial, mesenchymal and lymphoid origin.

The study of molecular pathogenesis of SGTs is a challenging task because of the rarity and histopathological diversity of these malignancies.

We are collecting STG samples with different hystotypes in order to characterize cytogenetic and genetic alterations to better understand molecular pathogenetic mechanisms, to correlate anomalies with clinico-pathological data and to identify potential diagnostic and prognostic markers.

Comparative genomic analysis metaphase based performed in ten samples with different hystotypes (eight adenoido-cystic samples-ACC, one epi-mioepothelial ME, one low-grade pleomorphus adenoma-PLGA) showed heterogeneity.

Mutational analysis of p14/ARF, p16 ink4a, TP53, PTEN, H-RAS, K-RAS, N-RAS, BRAF and MAK2 has been performed in three ACC, one ME and one PLGA; novel mutations in HRAS, p14 /ARF and p16 nk4a have been identified.

The correlation of our findings with clinical-pathological data and a comparison with literature data will be reviwed and discussed.

This work has been funded in the frame of the Project "Salivary gland tumors: different approaches to identify genetic and prognostic markers" Fasc 7GR1, Programma di collaborazione ISS-NIH, Area Malattie Rare.

A GENOME WIDE NON-SYNONYMOUS SNP SCAN OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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Background. Only 15% ALS patients have a family history, the remaining cases occurring sporadically in the human population. While familial ALS is well characterized with several causative genes identified to date, the genetics of sporadic ALS is poorly understood. In the past year three independent Genome-Wide Association (GWA) studies found no single gene strongly associated with susceptibility to ALS suggesting a complex interaction between environmental factors and many susceptibility genes of small effect best describes this disease. One such susceptibility gene of small effect recently nominated by a GWA study from different populations of European origin is Dipeptidyl-Peptidase 6 (DPP6) which slightly increases the risk of developing ALS (OR 1.3).

Aims. To confirm the association of DPP6 with ALS phenotype we tested the candidate polymorphism rs10260404 in an Italian population.

Methods. The Italian cohort included 907 cases and 1019 healthy controls collected by the first "Italian ALS Consortium" created by the collaboration of several Neurological Centres located in North Italy. All the DNA samples have been gathered at the Italian Auxologico Institute in 96-well bar-coded plates and quantified with PicoGreen fluorescent reagent (Invitrogen). Clinical information about ALS patients has been placed in a shared database according to the International form already used for the US and UK GWA studies so that genotype-phenotype correlations will be easily inferred from this database. All ALS cases had any recorded family history were previously screened for Zn/Cu superoxide dismutase1, alsin, angiogenin and TAR DNA binding protein genes.

Results. Assuming an OR of 1.3 with a causative allele frequency of 0.44, as described by the authors, we have >99% power to detect an association with a p value of 0.05.

Our preliminary data show no evidence of association of rs10260404 SNP with susceptibility to ALS (Fisher's exact chi-square test p=0.8). Moreover, genotype scores were tested for Association with Age at Onset (AAO), available for 743 cases (457 males, 286 females). AAO ranged from 15 to 84 years with mean 55.22 and median 57 years (sd 13.5). Kaplan-Meier survival analysis showed no significant association (Log Rank chi-squared 3.72, p 0.155, 2df) between variant rs10260404 genotypes and AAO and no differences for genders were detected.

Conclusion. Polymorphism rs10260404 in DPP6 gene, as other variants reported to be associated with sporadic ALS, has failed to be replicated in a different population. These

results highlight the genetic heterogeneity of sporadic ALS even within European populations. The same conclusions have been drawn for many complex diseases and emphasize the importance of large sample sizes and international collaborations for a meta-analysis of different studies.

NEUROLOGICAL IMPAIRMENT IN NIEMANN-PICK C DISEASE: A STUDY ON THE ROLE OF EXCITATORY NEUROTRASMITTER RECEPTORS AND IDENTIFICATION OF PERIPHERAL CELLULAR BIOMARKERS

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Changes in the functioning of neuronal plasma membranes are good candidates in the research of Niemann-Pick Disease type C (NPDc) pathophysiogenetic mechanisms. The loss of a correct dynamic of cholesterol-sphingolipids-enriched microdomains in the neuronal and glial plasma membrane, caused by an imbalance in the lipid trafficking due to NPDc gene mutation, might have a key role in neuronal dysfunction and the consequent clinical pathologies. Our previous results suggest that changes in the plasma membrane cholesterol content are particularly important in affecting lipid rafts, regulators of glutamate receptor functioning. We therefore evaluated through several experimental approaches whether the physiological properties and the neurotransmission of NPC neurons show differences from what observed in the Wild Type (WT).

To this aim acute brain slices, primary neuronal cell cultures and synaptosomal preparation from WT and NPC mice, a well-established mouse model for the Niemann-Pick type C disease, were used; in some experiments the Methyl-beta-Cyclodextrin (MbetaCD), a molecule that dissolves the hydrophobic core of lipid rafts, was perfused. The electrophysiological data suggest an impairment of the excitatory neurotransmitter receptors, since a different response to kainic acid perfusion was observed: in fact in hippocampal slices from NPC mice the excitotoxic effect properly described for the WT slices was lacking.

These data are in agreement with the results of WT MbetaCD-treated slices which respond to kainic acid application that partially resembles the NPC slices trend, confirming that lipid rafts manipulation counteracts kainate effect. Moreover the induction and maintenance of NMDA-dependent LTP in the CA1 region of NPC hippocampal slices were significantly reduced. The electrophysiological results are supported by data outcoming from cell culture experiments on excitatory aminoacid-induced intracellular calcium increase. Indeed, application of both NMDA and kainic acid in WT cell culture treated with MbetaCD significantly reduced calcium influx. Moreover, results from Western Blot analysis on synaptosomal membrane fractions revealed that levels of GluR6/7 kainate

receptor subunits were about 30% reduced in synaptosomes from NPC slices as compared to wild-type.

In order to develop a method for a rapid and suitable diagnosis, we used some fibroblast cell lines obtained from patients. Cell lines have been maintained in culture and the lipid composition was determined with standard procedures. Finally, we used different strategies to correlate sphingolipid/cholesterol membrane content with the pathological status of the fibroblast donor, based on the ability of some toxins to bind cell lipids. The results obtained encourage more detailed studies.

GENETIC, MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF COCKAYNE SYNDROME, A RARE TRANSCRIPTION/REPAIR DEFECTIVE HEREDITARY DISEASE

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Cockayne Syndrome (CS) is a genetically heterogeneous disease characterised by precocious ageing and progressive physical and mental impairment. To improve prevention and treatment of CS and to gain insights into its molecular and functional basis, our research has been focused on the following aspects:

- Cellular, genetic and molecular characterisation of newly identified patients (PI: M. Stefanini). The alterations in the cellular response to UV light typical of CS were detected in four out of six newly referred CS cases. Two patients were assigned to the CS-A group and one to the CS-B group. The fourth case was defective in the still-unidentified gene responsible for UV-sensitive syndrome, a recently recognized disorder showing only mild cutaneous symptoms. Cells from this patient represent a valuable material to verify the relationship between developmental alterations and sensitivity toward oxidative stress. Furthermore, we have characterized at the molecular and biochemical level four CS-B cases and we have collaborated to the establishment of the incidence for CS in Western-Europe (2.7 per million livebirths).

- Clarification of the functional bases of the altered response to oxidative stress of CS cells (PI: E. Dogliotti). We have demonstrated that CS-A cells are hypersensitive to oxidizing agents and accumulate 8-oxoguanine upon oxidative stress. In addition, CS-A fibroblasts showed an increased sensitivity to inhibitors of poly(ADP-ribose) polymerase, a nuclear enzyme that signals the presence of DNA damage and is implicated in the repair of DNA Single-Strand Breaks (SSB). Accordingly, CS-A cells accumulated DNA-SSB induced by hydrogen peroxide and methylmethanesulfonate. These findings provide the first evidence of the involvement of CSA in the processing of SSB and suggest that lesions and/or mechanisms other than 8-oxoguanine accumulation may contribute to neurodegeneration.

- Oxidative DNA damage repair defect in CS and its complementation by heterologous repair proteins (PI: G. Frosina). We have investigated whether the oxidatively damaged dna repair defect in CS might be corrected by heterologous DNA repair proteins, such as the *E. coli* Formamidopyrimidine-DNA Glycosylase (FPG). This protein has a broad substrate

range, being able to repair AP sites, 8-oxopurines and fapy purines. Expression of FPG totally reversed the 8-oxoguanine repair defect in CS cells. hence fpg may be a suitable candidate for relieving the 8-oxopurine repair defect in cs patients. similar studies are under way with the *E. coli* endonuclease III (NTH) protein that efficiently repairs oxidized pyrimidines. these complementation studies may shed light on lesions relevant for the CS phenotype and offer new therapeutic options.

A DOUBLE-BLIND PLACEBO-CONTROLLED CLINICAL TRIAL ADDRESSING THE INHIBITION OF PDGFR PHOSPHORYLATION AS A CANDIDATE PATHOGENETIC TREATMENT OF SYSTEMIC SCLEROSIS

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Disease modifying therapy for Systemic Sclerosis (scleroderma, SSc) is an unmet medical need. Excessive oxidative stress has been implicated in the natural history of the disease, since ROS generation is a putative bridge between the Ha-Ras and growth-factor activated extracellular signal-regulated kinases 1/2 (Ha-Ras, ERK1/2): this circuit is amplified in scleroderma fibroblasts.

The recently identified stimulatory autoantibodies against PDGF receptor possibly provide a link between autoimmunity and fibrosis in SSc: they induce ROS production via Ha-Ras and ERK1/2 recruitment and are ultimately responsible for SSc fibroblast activation via the intracellular kinases system, with collagen overproduction.

Inhibition of this pathway is therefore a candidate strategy for molecular intervention in SSc patients. Imatinib mesylate, the standard therapy for chronic myeloid leukemia, is a specific inhibitor of ABL kinases, which normally phosphorylate the PDGF receptor. Recent reports suggest its usage in the Bleomycin (BLM)-induced lung fibrosis model. The objective of the present study is to treat scleroderma skin fibrosis using a therapeutic strategy based on this pathogenetic mechanism. The study is a multicentic, randomized double-blind, placebo-controlled trial.

Thirty SSc patients with refractory disease (worsening of skin involvement or visceral damage despite adequate immunosuppressive and vasoactive therapy at standard doses for at least 3 months) will be randomly assigned to receive or not Imatinib 200 mg *die* orally

for 6 months (+6 months of follow-up) in addition to the conventional treatment. The preliminary authorization for the study has been recently obtained by the Ethical.

Committee of the San Raffaele Scientific Institute, the coordinating center, and other authorization requests are pending. Primary outcome measures include the evaluation of: i) the safety of Imatimib administered in low dose in SSc patients; ii) the efficacy of imatinib to improve sclerodema skin disease by assessment of skin thickness evaluated by the modified Rodnan skin score, which reflects skin fibrosis; iii) the amelioration of the quality of life and the patient physical and emotional well being, evaluated by HAQ (Italian version) and SF-36 score. Secondary outcomes will be: i) the capacity of the drug to revert *in vitro* the functional alterations of the SSc fibroblasts (Ros generation, collagen production, PDGFr phosphorylation) appraised on skin biopsies obtained prior to treatment, at the end of treatment and after 3 and 6 months of follow-up; ii) the efficacy of imatinib on pulmonary interstitial disease, assessed by pulmonary function test, Diffusing Lung Carbon Oxide, six minute walk test, and high-resolution CT scan.

FOR A DEFINITION AND A LIST OF RARE CANCERS IN EUROPE

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The burden of rare tumours is not known, both in Europe and in Italy. For this reason, two projects have been funded to estimate the basic epidemiologic indicators of frequency (incidence, prevalence and mortality) and outcome (survival) for rare cancers in Europe and Italy. The first step of both projects is to provide a definition of rare malignancies and an exhaustive list of them.

Our contribution will describe the methodology followed to reach an international agreement on a definition and list of rare cancers that will permit to identify the rare entities for which the indicators will be calculated.

The International Classification of for Oncology (ICD-O) is the tool utilised for the definition of the tumour entities. Population-based cancer registries are the basis for both the calculation of the indicators and the definition of the threshold. An international group of experts was formed in order to reach an agreement for an operative definition and a definitive list of rare malignant tumours. Three important elements were discussed within the experts: the indicator of frequency, the entity and the threshold.

With respect of indicator of frequency, the group of experts, mainly clinicians from the ESMO Faculty, agreed that incidence is the most appropriate indicators for rare cancers. Tumour entities were defined by combinations between morphology and topography. The provisionally identified threshold is 3/100,000/year range under which a tumor might be reasonably considered as rare. Incidence was calculated as crude rate from pooled European data.

Other criteria such as age, sex, clinical presentation and biological features were not considered for the definition of the rare entities. The final definition and list will circulate during the summer among the experts and all the partners of the projects in order to reach a final agreement.

ADIPOSE TISSUE-DERIVED STEM CELLS FOR THE TREATMENT OF MUSCULAR DYSTROPHY

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Duchenne Muscular Dystrophy (DMD) is a progressive X-linked muscle wasting disease, leading to early disability and death. DMD is caused by a mutation in the dystrophin gene that precludes the production of a stable protein, causing disruption of muscle contractile structures.

Therapeutic approaches to DMD aim at rescuing muscle damage by delivery of cells able to differentiate into skeletal muscle. Skeletal muscle-derived progenitor cells represent one choice because of their intrinsic myogenic potential. Unfortunately, these cells are recovered in low number from DMD muscle biopsies and are poorly expandable *in vitro*. Thus it is important to identify alternative muscle progenitor sources able to contribute to skeletal muscle regeneration.

Adipose Tissue (AT) provides a uniquely abundant and accessible source of multipotent cells. We have recently shown that in addition to mesenchymal stem cells, adipose tissue contains a subpopulation of cells, referred to as Adipose Tissue-Derived Autonomously Myogenic Cells (AT-AMCs), which are able to spontaneously differentiate into contractile skeletal myotubes. When transplanted *in vivo*, AT-AMCs participate in the formation of new muscle fibers and may contribute to the replenishment of the muscle progenitor pool.

In this study we report that AMCs are not restricted to adipose tissue but can be found in a variety of adult mouse muscle-devoid tissues such as pancreas, spleen and stomach. Immuno-magnetic selection procedures indicate that AMCs from adipose and from other tissues derive from Flk-1^+ progenitors. Individual clones of myogenic cells from non-muscle organs are morphologically and functionally indistinguishable from skeletal muscle-derived primary myoblasts.

Moreover, they can be induced to proliferate *in vitro* and are able to participate in muscle regeneration *in vivo*. Thus, we provide evidence that fully competent myogenic progenitors can be derived from the Flk-1^+ compartment of several adult tissues that are embryologically unrelated to skeletal muscle.

Pitx2 CONTROLS BETA-CATENIN mRNA STABILITY

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The Axenfeld-Rieger Syndrome (ARS) is a severe genetic desease which develops before birth. ARS is an autosomal-dominant disorder characterized by ocular anterior chamber anomalies causing glaucoma, dental hypoplasia, craniofacial dysmorfism, umbilical stump anomalies, and abnormal cardiac, limb, and pituitary development. Mutations of the paired-like homeodomain transcription factor 2 gene, Pitx2, represent the most common genetic signature of ARS. We have shown that targeted deletion of Pitx2 gene in mouse recapitulates the human desease features and that Pitx2 is a crucial modulator of cell proliferation in heart and pituitary gland.

Furthermore, we have demonstrated that the Wnt/beta-catenin pathway controls Pitx2 expression both at the transcriptional level and at the level of the mRNA turnover. Here we demonstrate that Pitx2 overexpression in alfaT3 pituitary cells causes increased expression of both Akt1 and Akt2 determining the activation AKT signaling pathway and, as a consequence, increased expression of beta-catenin. Beta-catenin plays an essential role in several biological events including cell fate determination, cell proliferation, and transformation. Here we report that beta-catenin is encoded by a labile transcript whose half-life is prolonged by AKT signaling pathway. AKT phosphorylates the mRNA decay-promoting factor KSRP at a unique serine residue, provokes the unfolding of KH domain 1, induces KSRP association with the multifunctional protein 14-3-3, and prevents KSRP interaction with the exoribonucleolytic complex exosome. This impairs KSRP's ability to promote rapid mRNA decay. Our results uncover an unanticipated level of control of beta-catenin expression pointing to KSRP as a required factor to ensure rapid degradation of beta-catenin in unstimulated cells. In conclusion, Pitx2 overexpression, through inhanced AKT expression, determines an accumulation of beta-catenin transcript.

PATHOGENETIC ROLE OF ISOLATED HUMAN TSC2 SMOOTH MUSCLE CELLS AND ITS PHARMACOLOGICAL CONTROL. NOVEL PERSPECTIVES IN TSC AND LAM

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Tuberous Sclerosis Complex (TSC) is a syndrome caused by mutation in TSC1 or TSC2 genes. From the angiomyolipoma of a TSC2 patient, we isolated an homogenous population of smooth muscle-like cells (TSC2-/- ASM cells). The EGF-dependent proliferation of TSC2-/- ASM cells was analysed following transfection of TSC2 gene.

This procedure eliminated the EGF-requirement for growth and decreased the phosphorylation of Akt, PTEN, ERK and S6. Anti-EGFR antibody reduced markedly the constitutive phosphorylation of S6 and ERK. Exposure of TSC2-/- ASM cells to rapamycin reduced the rate proliferation, when added at plating time. Akt function in TSC2-/- ASM cells was poorly inhibited by PI3K specific inhibitors, and TSC2-transfection instated normal Akt control by PI3K.

The effects on lung structure and function was studied in immunodeficient nude mice. Cells were applied in anesthetized mice by endonasal application, 5 droplets containing 50,000 cells each were singly applied with a separation interval between the consecutive applications of 5 minutes. TSC2-/- human smooth muscle cells penetrated the lung parenchyma within 48 hours of their application and reached also the uterus via the lymphatic system. After endonasal application, TSC2-/- cells survived and proliferated in lung parenchyma creating a condition that somewhat resembled LAM.

The lungs were invaded by lymphatic vessels, and in 6 months the extent of their diffusion in lungs was 10 fold higher than normal. The penetration and expansion of the lymphatic vessels driven by the correlate with a destruction of lung parenchyma. Treatment with rapamycin and EGF was begun 6 months after cell administration. Preliminary results suggest that treatment with the antibody to the EGF receptor eliminated most of invading TSC2-/- cells and promoted the regression of the lymphatic vessels. Differently rapamycin resulted ineffective. In conclusion our work suggests that anti-EGFR treatment causes the death of human TSC2-/- cells both *in vitro* and *in vivo*, and partially reverses their phenotype, while rapamycin has a cytostatic effect only when added at plating time and *in vivo* may not be effective. The introduction of the TSC2 gene into human TSC2-/- cells normalizes proliferation and phenotype of these cells.

We have recently isolated, from a patient angiomyolipoma, another pure TSC2 smooth muscle cell population with the second hit on the TSC2 gene originated by means of an epigenetic mechanism.

We have also purified from chyle and lungs of three LAM patients, from Italy, Spain, and UK, pure LAM TSC2-/- smooth muscle cells, now under characterization.

MESENCHYMAL STEM CELLS FOR THE TREATMENT OF TIBIAL CONGENITAL PSEUDARTHROSIS ASSOCIATED WITH TYPE I NEUROFIBROMATOSIS

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Type 1 Neurofibromatosis (NF1), the most common single gene disorder found in humans, is the main cause of Tibial Congenital Pseudarthrosis (TCP). The treatment of TCP consists on repeated surgical procedures which often fail with the inevitable outcome of severe disability. The use of autologous bone Marrow Stromal Cells (MSC) has been recently proposed, because the MSC pool contains osteogenic precursors, that in turn may enhance bone repair. In the 1st year of the project our preliminary results showed that the osteogenic potential of MSCs was higher in Iliac Crest (IC-MSC) than in the Pseudarthrosis site (P-MSC), suggesting that the autologous MSC transplantation may be a promising strategy for improving bone consolidation where traditional techniques fail. In the 2nd year we planned to complete the sample collection, and to test whether bone microenvironment may affect the osteogenic potential of IC-MSC. Bone fragments from pseudarthrosis lesion were collected from each patient in order to obtain primary osteoblast culture (HOB). HOB and MSC from the same individual were used in a co-culture system in order to mimic the interaction between microenvironment (HOB) and osteoprogenitors (MSC). We enrolled 5 patients affected by NF1 and TCP (TCP-NF1+; 4M/1F, age 0.7-16 yrs), 6 patients affected by TCP without NF1 (TCP-NF1-; 5M/1F, age 2.6-18 yrs), and 2 healthy male donors (5 and 7.5 yrs). Both IC-MSC and P-MSC were cultured in osteogenic medium containing α-MEM, 10% fetal bovine serum (FBS) or 10% autologous serum (AUT), 10^{-8}M dexamethasone, and 50 μg/mL ascorbic acid. The ability to generate bone-forming cells was tested by measuring cell proliferation, alkaline phosphatase activity, mineral nodule formation, and gene expression, and it was higher in IC-MSC than in P-MSC cultures, even though significant differences were observed only in TCP-NF1+. In these patients, the *in vitro* mineralization of IC-MSC was reduced by AUT serum, since the number of mineral nodules was similar to that observed in P-MSC. Generally, the osteogenic potential of IC-MSC was lower in patients than in healthy donors. The co-cultures were performed in 7/11 patients, and the preliminary results suggested that the osteogenic differentiation of IC-MSC was affected by HOBs derived from pseudarthrosis, but the proliferation seemed to be inhibited only in TCP-NF1+. These findings imply that bone microenvironment could influence negatively the success of the autologous MSC transplantation, and further studies have to be performed to better understand the mechanism by which that occurs.

The study was performed with the collaboration and contribution of Istituto Superiore di Sanità (Programma Italia-USA "Malattie Rare") and "Io ci sono" Association.

P. NEURAL TUBE DEFECTS AND FOLIC ACID: AN INTEGRATED, EVIDENCE-BASED APPROACH TO PRIMARY PREVENTION IN THE ITALIAN CONTEXT

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Anencephaly, encephalocoele and spina bifida are malformations occurring during embryonic development and neural tube closure which occurs between the 17th and 30th day after the conception. These malformations are of different severity however they share common pathways and are considered together as Neural Tube Defects (NTDs).

Folic Acid (FA) (or B9 vitamin) is a vitamin essential for metabolism of sulphur-amino acids and nucleic acids. Many studies support the role of FA in the primary prevention of NTDs, so far different approaches to increase FA intake include promotion of healthy dietary habits, periconceptional supplementation and fortification of staple foods, have been developed.

Within our project we focused on the following topics:

- Development of a model for primary prevention of NTDs based on FA and folate supplementation at appropriate dose and at the correct time of pregnancy. This model, validated in a local district of Regione Marche, will be also implemented in ten more health districts (at list 70-100,000 inhabitants) and will be focused on active offer of health promotion on acid folic supplementation.
- Two-hundred and eighteen questionnaires on folic acid intake before and during pregnancy (for cases, mothers of twins and controls, mothers of singletons) were sent by mail to mothers of twins during the last week of June 2008. After 3 weeks we have received back 50 questionnaires from cases and 8 from controls. About 200 kit for saliva collection were also sent together with the questionnaires and 56 we received back after 3 weeks. The second part of the sample (about 300 hundred cases and controls) will be contacted in September 2008.
- A surveillance on 620,000 births from 1996 to 2006, performed by National Centre for Rare Diseases (NCRD) and supported by Birth Defect Registries was performed in order to evaluate the impact of folic acid supplementation on the frequency of NTD on this sample. The trend analysis was not significant and an heterogeneous trend was observed. More detailed statistical analyses will be performed in the next few months.
- Evaluation of the evidences on the correlation between biomarkers of folate status and adverse effects of FA in populations in which generalized FA flour fortification has been performed.

Folate levels in serum or in plasma associated with an initial increment of adverse effects in vulnerable subjects (middle aged or elderly) are in the >25 nmol/l range. Since such levels

are reachable in populations in which general food fortifications is being performed, a precautionary approach is recommended toward such procedure, at least in anticipation of further studies. On the other hand, in order to prevent neural tube defects it is advisable to effectively support strategies such as the promotion of a more proper nutrition and the periconceptional supplementation.

Our effort for the future will be based on the implementation of strategy for the promotion of FA supplementation (including targeted actions towards potentially vulnerable groups) thus estimating whether their influence on NTDs trend prevalence. Furthermore, the strength of association between the FA supplementation and multiple pregnancy will be estimated.

TGFBR1 AND TGFBR2 GENE MUTATIONS IN LOEYS-DIETZ AND THORACIS AORTIC ANEURYSM DISSECTION SYNDROMES

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Mutations in the TGFBR1 and TGFBR2 genes cause the autosomal dominant Loeys-Dietz Syndromes (LDS). Based on the presence or absence of cranio-facial traits the syndromes are classified as LDS 1 and LDS 2, respectively.

We performed clinical and molecular characterisation of 92 members of 29 families whose probands were addressed to our attention with the suspected diagnosis of Marfan Syndrome (n=25) and familial Thoracic Aortic Aneurysm and Dissection (TAAD) (n=4). Probands and realtives underwent genetic counselling, multidisciplinary clinical and imaging evaluations and molecular analysis of the FBN1 (n=25) TGBFR1 and TGRBR2 genes (n=29).

We found 31 TGFBR1&2 mutations in 29 probands; two carried a double heterozygosity, one inherited and one *de novo*. 23 relatives carried the corresponding proband's mutation. Based on phenotypical ground 29/52 mutations carriers were diagnosed with LDS1 and 23 with LDS2.

The two syndromes shared aortic aneurysm (93% and 91%), high rate of dissection (27% and 38%), arterial tortuosity (100%) and, at a minor rate, aneurysms of other vessels (45% and 29%) and skin/integumental traits. The disease was *de novo* in 53% of the probands with LDS1 and 4% of probands with LDS2. The mean age at first diagnosis was 18.44±17.14 (<1-46) and 40.78±15.56 (14-68), at first surgery 24.53±14.17 (1-46) and 44.61±14.55 (18-69), with 42 interventions in 28 patients. Congenital heart defects and cranio-facial, skeletal, ocular, and nervous system traits typically recurred in LDS1.

The LDS1 and LDS2 severely affect the cardiovascular system, with arterial tortuosity as common maker shared by the two syndromes.

THE SIGNIFICANCE OF SURGICAL TECHNIQUES EVOLUTION IN THE TREATMENT OF DEFORMITIES ASSOCIATED TO RARE DISEASES: SCOLIOSIS IN PRADER-WILLI SYNDROME

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Introduction. The incidence of spinal deformity in children with Prader-Willi Syndrome (PWS) is high, with 86% of these patients found to have a significant structural scoliosis; however, reports describing surgical treatment for this syndromic deformity are rare.

Method. We report a review of 6 case series that underwent surgical procedure for scoliotic curve correction in our Institute. Children's average age at the time of the first surgical procedure was 12.8 (min. 10, max. 14.5) years. Clinical evaluation revealed typical PWS phenotype characteristics in all cases; 4 individuals had a karyotype confirmed diagnosis. The first patient received a hybrid stainless steel instrumentation with sublaminar wires, hooks and a couple of distally inserted pedicle screws. We used hooks and screws in the second patient, while the latter 4 ones ware treated exclusively with titanium pedicle screws instrumentation. One of these 4 latter cases, initially underwent dual rod instrumentation surgery and received definitive fusion after an 18 months period. The other 5 patients underwent posterior arthrodesis procedure by first operation. Major structured curves, with Cobb angles rating from 55° to 96° (mean 80.5°) pre-op, presented a post-op average correction of 48.5° (min. 24°, max. 65°).

Results. A single major complication was registered (rate 16.6%): an intra-operatory paraparesis, followed by complete regression after the operation (total removal of the instrumentation was necessary), representing the only case where spinal fusion was not achieved. We had 1 minor complication (rate 16.6%): detachment of a instrumentation bar located distally 3 months after operation, corrected with revision and one level caudal extension. Mean follow-up period was 3 years and 5 months (min. 11 months, max. 9 years). Follow up data revealed that correction was preserved in 5 cases, with a correction loss of only 5.5° in major structured curves and 3.6° in minor structured curves. Lateral deviation of the cervical spine emerged in 1 case and hyper-kyphosis of the upper thoracic segments was observed in another one.

Conclusions. Instrumentation with posterior pedicle screws exclusively and posterior arthrodesis allows to obtain immediate solid correction of the deformity, it reduces risks and peri-operatory complications and improves the post-operatory course of these patients, allowing an immediate mobilization, that does not necessitate orthesis of any kind. The choice of using dual rod technique must be carefully evaluated in each single case, keeping in mind that this pathology relates to low mean high stature as well as potential effects of a GH treatment.

MOLECULAR ANALYSIS OF ARSA AND PSAP GENES IN TWENTY-ONE ITALIAN PATIENTS WITH METACHROMATIC LEUKODYSTROPHY. IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF 11 NOVEL ARSA ALLELES

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Metachromatic Leukodystrophy (MLD), the demyelinating disorder resulting from impaired sulfatide catabolism, is caused by allelic mutations of the Arylsulfatase A (ARSA) locus except for extremely rare cases of Saposin-B (Sap-B) deficiency. We characterized twenty-one unrelated Italian patients among which seventeen were due to ARSA activity deficiency and 4 others resulted from Saposin-B defect. Overall, we found 20 different mutant ARSA alleles and 2 different Sap-B alleles.

The new eleven ARSA alleles consisted of 8 missense mutations (p.A18D, p.D30H, p.A212P, p.N282S, p.K302N, p.Y376N) including two in cis (p.R217H; p.R370W); 2 nonsense mutations (p.W124X and p.E307X); 1 in-frame deletion (c.409_411delCCC) leading to the loss of the Proline 137 (p.P137del); 1 splice-acceptor-site mutation (c.1102-3C>G) affecting mRNA processing.

The functional relevance of the intronic mutations (c.1,102-3C>G) was determined by carrying out reverse transcriptase-polymerase chain reaction analysis that revealed two anomalous transcripts (complete skipping of exon 7 and a partial loss of exon 7).

To address the question of the potential impact of the novel codon replacements on protein function we performed *in vitro* expression experiments. Wild type ARSA cDNA and the mutant constructs (p.A18D, p.D30H, p.A212P, p.N282S, p.K302N, p.Y376N, p.R217H and p.R370W) were transiently transfected in COS-7 cells. All tested mutants expressed extremely low residual ARSA activity. Additionally, to evaluate a possible cumulative effect on enzyme activity, the two in-cis mutations (p.R217H; p.R370W) were analyzed both singly and in combination showing that the two mutations, in combination, drastically reduced the enzymatic activity.

Finally, to further understand the consequences of these new ARSA alleles at the protein level, we modelled the amino acid changes into the three-dimensional ARSA structure. According this virtual model, p.D30H and p.N282S are predicted to disrupt the metal binding site; the change p.K302N, altering the local charge, is though to prevent the correct positioning of a sulphate group of the substrate and consequently its hydrolysis. Additionally, the mutations p.A212P, p.R217H and p.Y376N might compromise the local

folding of the protein, whereas the replacement of Y376N is expected to leave a solvent-accessible surface area in proximity of a cluster of hydrophobic residues with consequent destabilization and/or incorrect folding of the protein.

The present study is aimed at providing a broader picture of the molecular basis of MLD Italian population. Our findings demonstrate and confirm the necessity of a comprehensive evaluation, based on a range of diagnostic procedures including neuroradiological, neurophysiological, biochemical and molecular tests, to shed light on the underlying pathological causes of MLD.

FAMILY BASED TRANSMISSION ANALYSIS OF GENETIC MARKERS IN CLASS I AND CLASS III HLA REGION IN SARDINIAN CHILDREN WITH AUTISTIC SPECTRUM DISORDERS

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Introduction. Autism Spectrum Disorders (ASD) are a group of behaviourally defined neurodevelopmental syndrome with onset in childhood. ASD with a wide variety of both genetic and non genetic causes.

The wide phenotypic variability of the ASDs likely reflects the interaction of multiple genes within an individual's genome and the existence of distinct genes and gene combinations among those affected. In a recent family based study we strongly concurred with the findings of other authors, which indicate that the basis of the strongest MHC associations appears to be an extended haplotype that comprises a relatively constant sequence of DNA over a large multi-region of the MHC, inclusive of, but not limited to, the HLA-B region, through the HLA DR region.

On these basis we went into more depth of our previous study, scanning by microsatellite and SNPs analysis an approximately 6 Mb region spanning the HLA class I region, from HLA-B to HFE gene. Four microsatellites (MIB, d6s265, MOGc and d6s2239) and three SNPs (2 SNPs in position -308 and -238 in the promoter of the TNF α, and the SNP rs2857766 (V142L), (in the exon 3 of the MOG gene) were analysed.

Methods. Thirty-seven families of Sardinian ancestry, all of whom had at least one autistic child, were enrolled and genotyped to evaluate microsatellite and SNPs markers association to ASD. We used an intrafamilial case control method of analysis (AFBAC Affected Family-BAsed Controls,) and furthermore to assess preferential allelic transmission from heterozygous parents to affected offspring, the TDT test was performed.

Results. Both AFBAC evaluation and TDT analysis evidenced a positive association of D6S265(220) microsatellite (located 115Kb centromeric of HLA-A) with ASD(p_y<0.01; (OR=4.92, IC(95%):1.5-21.0), while MOGc(117) (262Kb telomeric of HLA-A) resulted less transmitted to ASD children (py=0.014, OR=0.36, IC(95%):0.16-0.83). Also MIB(346) allele was less transmitted to ASD children (p_y=0.008; OR=0.09, IC(95%):0-0.7)

No differences between transmitted and not transmitted alleles were revealed for all the other analysed markers.

Conclusions. These results give further support to our previous data, indicating the HLA region as a genetic locus involved in ASD development and furthermore focus a more restricted region of genetic markers of susceptibility (d6s265) or protection (MOGc, MIB), to be scanned further.

SOX7 AND -17 FUNCTION AS MODIFIERS OF THE LYMPHANGIOGENIC ROLE OF SOX18. NEW INSIGHTS IN THE PATHOGENESIS OF THE HUMAN SYNDROME HYPOTRICHOSIS-LYMPHEDEMA-TELANGIECTASIA

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In the previous work within this program, we found that the transcription factor SOX18 is required for activating endothelial transcription of Prox1, thus initiating the lymphatic endothelial program of development from blood vascular precursors.

Mutations in SOX18 result in lymphatic dysgenesis in the human syndrome Hypotrichosis-Lymphedema-Telangiectasia (HLT). However, the phenotype of Sox18-null mutant mice produced by gene targeting varies dramatically depending on the background strain. In the following work we found that two closely related Group F SOX factors, SOX7 and SOX17, are upregulated in the absence of SOX18 during the genesis of the lymphatic vasculature, on a mixed genetic background but not on a C57BL/6 background.

Like SOX18, SOX7 and -17 are also able to activate Prox1 transcription *in vitro*, in cultured cells and in transgenic mice. Our results indicate that SOX7 and -17 act as strain-specific modifiers of the lymphangiogenic role of SOX18. These data may explain the variability of clinical manifestations of the rare human syndrome HLT.

NEW FINDINGS FROM MECP2-308 AND KFL7 MICE AS MODELS OF MENTAL RETARDATION

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Longitudinal behavioural characterization in a mouse model of Rett syndrome (RTT): mutations in the X-linked methyl-CpG- binding protein 2 (MeCP2) gene account for RTT, a severe neurodevelopmental disorder and genetic cause of mental retardation (1/15,000 girls). Clinical characteristics only appear between 6-18 months of age. In MeCP2-308 mutant male mice, a picture of impaired communicative capacity (ultrasound emissions) as well as subtle anomalies in spontaneous general movements were evidenced early after birth, during the so-called pre-symptomatic phase. Further at adulthood, these mice exhibited increased anxiety-like behaviours. Present results suggest that an increased attention devoted to the characterization of the pre-symptomatic phase can provide precocious biomarkers of RTT and a window of opportunities on which potential therapies could be tested. We thus investigated the efficacy of postnatal supplementation with choline (25 mM; until weaning), a vitamin of the B-complex and acetylcholine precursor in neurons. Reduced locomotion and increased emotionality were evidenced in adult mutant mice, compared to wt controls. Remarkably, choline treatment largely compensated these behavioural alterations. Present findings suggest that choline from early after birth rescues adult behavioural symptoms in mutant offspring. To probe the functional status of central cholinergic system, mice were challenged with the cholinergic muscarinic antagonist, scopolamine (2 mg/kg). The expected hyperactivity profile was not observed in mutant mice, thus revealing an underlying reduced cholinergic tone. This cholinergic alteration appears in agreement with previous observation in RTT human brains and paves the way to further therapeutic approaches. Analysis of the role of Kruppel-like factor 7 (Klf7), a transcription factor in the CNS and a candidate brain developmental gene associated with altered neuronal plasticity and mental retardation. Klf7 null mice die at birth and share severe defects in olfactory bulbs development and dendritic differentiation. Klf7 gene silencing was found to lead to impaired functional cardiomyocytes and delayed neuronal outgrowth. Klf7 KO murine embryonic fibroblasts showed abnormal differentiation to adipocytes and to osteocytes. CNS tissues from KO mice revealed a reduction of dopaminergic markers in the midbrain and the olfactory bulbs. Assessment of the human genomic features of the homologue counterpart of Klf7 indicated this gene to span approximately 86 kbs and to be composed of 4 exons. The nucleotide sequence studies established the existence of two KLF7 transcripts and to design the exonic-intronic structure. A 98% identity value between the human and mouse proteins was found. The presence of conserved sequence tags is currently in progress.

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MEASUREMENT OF NAD(P)H AUTOFLUORESCENCE BY VIDEO-MICROSCOPY IN *EX-VIVO* AND *IN VITRO* MODELS OF AMYOTROPHIC LATERAL SCLEROSIS (ALS) AND DISEASES CONNECTED WITH MITOCHONDRIAL CONDITIONS

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Background. Abnormal number, structure and localization of mitochondria in motor-neurons and skeletal muscle, and findings of altered respiratory chain enzyme activities were described in ALS patients. In G93A mice, a well accepted ALS model, abnormal mitochondrial enzyme activities were reported, and conversely, agents that enhance energy metabolism extend lifespan and delay onset of symptoms; hence mitochondrial studies appear of great interest in ALS.

Specific aims of the investigation: i) to study bioenergetic defects in the initial stages of mSOD1-induced toxicity in G93A mice living brain slices; and ii) to set up methods aimed to study mitochondrial complex I function in cells other than neurons, which can allow an early detection of mitochondrial impairment.

Methods. NAD(P)H levels were measured by the autofluorescence video-microscopy technique. Part of the experiments were carried out according to previously reported techniques.

Results. i) Mitochondrial complex I activity was monitored in the *ex-vivo* primary motor cortex in three groups of mice (WT, human SOD, G93A) at the ages of 60, 90, 130 d. We evidenced age related consistent alteration in post-sinaptyc responces of NAD(P)H autofluorescence levels, following ultraviolet stimuli, in G93A brain slices versus controls; ii) Preliminary experiments aimed to measure NAD(P)H were also performed in cells cultures: NAD(P)H reduction by mitochondrial complex I (CX I) was evaluated in microglia and fibroblasts utilizing the CX I inhibitor rotenone; non-mitochondrial NAD(P)H reduction was evaluated in immortalized acute myeloid leukemia cells using the NADPH-oxidase inhibitor apocynin.

Conclusions. These results indicate a possible application of the technique to pathological models of human diseases.

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P. PROPOSAL FOR AN INTEGRATED APPROACH TO RARE DISEASES: A STUDY BETWEEN BASIC LABORATORY MODELS AND CLINICAL EPIDEMIOLOGY IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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Our project is addressed to Amyotrophic Lateral Sclerosis (ALS) epidemiology, collecting data on large populations, using different methods - Work-Packages (WP) 1-3 to pathogenesis at cellular and molecular level (WP 4).

WP1: The Italian National Registry of ALS. Centro Nazionale Malattie Rare, Istituto Superiore di Sanità.

The National Registry of ALS in collaboration with several regional registries (PARALS-Piemonte; SLALOM-Lombardia; SLAP-Puglie) have been activated and are collecting clinical and epidemiological data on ASL. Has been agreed on data set which includes the informations of the National Register Rare Diseases information and several additional items such as:

- diagnostic criteria El Escorial;
- spinal/bulbar onset;
- inheritance;
- twin status;
- natural history of disease (PEG, tracheostomy, Mechanic ventilation).

The National Registry of ALS is available online (www.iss.it/cnmr).

WP2: ALS and primary lateral sclerosis in Italy: prevalence and incidence from hospital discharge data. Unità di Statistica, Centro Nazionale di Epidemiologia, Istituto Superiore di Sanità.

For ALS we found about 1,360 cases hospitalized for the first time during 2003 and 1,390 hospitalized for the first time during 2004, corresponding to a mean incidence rate of 2.4 per 100,000 inhabitants. About 4,000 ALS patients were found alive at 1/1/2003 corresponding to a prevalence rate of 7.0 per 100,000 inhabitants. For Primary Lateral Sclerosis about 150 new hospitalizations were recorded in the biennia 2003-2004 corresponding to a mean incidence rate of 0.13 per 100,000 inhabitants. About 240 PLS prevalent cases were found, corresponding to a rate of 0.42.

WP3: Evaluation of genetic and environmental factors in a cohort of twins with ALS. Registro Italiano dei Gemelli, Centro Nazionale di Epidemiologia, Istituto Superiore di Sanità.

Possible twins are being identified linking the Italian Twin Registry to ALS patients lists provided by 3 ALS regional Registries and 14 diagnosis Reference Centres. So far, among 4,982 patients (60% males), diagnosed with "definite", "probable" or "possible" ALS since 1990, we ascertained 29 twins (none of them belong to the same pair) plus 18 patients whose twin status is under ascertainment.

Twenty-two ALS twins (15 males) are from same sex pairs and 7 (6 male) belong to unlike sex pairs. As regards living status, in 1 pair both twins (ALS twin and co-twin) are deceased, while in 15 pairs 14 ALS twins and 1 co-twin are deceased. Health status or cause of death of co-twins will be ascertained by a neurologist. Life exposure to putative ALS risk factors will be investigated through a co-twin control study. Collection of biological material from ALS and unaffected twins is also envisaged.

WP4: NAD(P)H transient technique in *ex-vivo* and *in vitro* models for the studies of Amyotrophic Lateral Sclerosis (ALS) and diseases connected with mitochondrial conditions. Farmacologia della salute della donna e del bambino, Dipartimento del Farmaco, Istituto Superiore di Sanità.

We evidenced a consistent alteration of NAD(P)H autofluorescence levels in G93A brain slices, and also in recycling pattern following ultraviolet stimuli in cellular models. These results indicate a new investigation procedure, in that our investigation equipment can be applied not only to *ex-vivo* brain slices, but also to peripheral, non-neuronal cells, and can give interesting information for the studies of metabolic conditions connected with mitochondrial diseases.

P. NEEDS OF PEOPLE INVOLVED IN RARE DISEASES

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Rare diseases represent about 10% of known human pathologies and affect a relevant part of the Italian population. Among the problematic experiences faced by the people dealing with these kind of pathologies, communication and relationships should draw particular attention. On this topic the National Centre for Rare Diseases of the "Istituto Superiore di Sanità" started a pilot project "Effective communication and counselling: Improving listening in rare diseases". This is a national, multicentric and pilot experience of cooperation among different subjects. The project of two years duration has the following goals: i) to identify the planning criteria for the communication process in rare diseases, and to improve the scientific and technical expertise; ii) to implement more diffused and comprehensive communicative procedures based on integration and participation models; iii) to favour a cooperative network exchange among people involved in rare diseases.

The study involves health workers working for the National Health Service and/or charities and implies four phases.

Phase 1: this phase is about setting up and actualising a qualitative study. This is achieved by activating focus groups made of health workers, representatives of associations of patients, patients themselves and their relatives. The objectives was to identify the real needs and the critical areas of management of rare diseases. Each focus group was made up of a facilitator, an observer and 10-12 participants had a duration of 90 minutes and was video-recorded upon explicit and informed consent.

Contents were typed on a computerised system and eventually were analysed in order to: i) identify areas of strength and critical areas in the communication; ii) identify the needs of those directly or indirectly involved in the field of rare diseases; iii) identify the information needed by the people affected by rare diseases.

Select forms of communication through which it was possible to supply useful information to patients, their families and to health workers. It has emerged by analysing the contents of the focus groups that the needs mostly expressed by the patients and their families are: i) to make their rights acknowledged by those involved in the subject; ii) to be heard by health workers and to get information in a comprehensible languafe; iii) to have a health coordinator who should be able to coordinate the work of different specialists and workers involved in the care and management of the patient. For the health workers the main needs are:

- continuous education;
- the possibility to be part of a network with the involvement of general practitioner;

– to share experiences and knowledge with professionals belonging to different areas;
– the availability of protocols and guidelines.

Furthermore, the compared analysis of different focus groups has shown some point of concordance about the need of making the communication between different subjects more effective with a greater listening attitude.

Phase 2: the indication obtained from the focus groups have allowed the elaboration of two different questionnaires: one for health workers and one for charities. Both had the goal of eliciting the needs for continuous education in the fields of rare diseases.

This investigation will obtain useful indicators for the setting up of a process of continuous education for both health workers and people involved in the work of the charities. Teaching strategies able to develop communication and relational abilities and update the technical and scientific knowledge of different subjects will also allow sharing different experiences for an effective cooperation among colleagues of singles services (group work) and among workers of different services and charities (working in a network).

Phase 3 and 4: for the following phases we plan the monitoring of the modified aspects of knowledge and behaviour of those who have taken part in the study through a self monitoring form. Finally, the data will be collected and analysed. Guidelines will be put in writing and there will be a proposal of implementation of the project nationwide.

Conclusions. The preliminary results give interesting hints about the needs of people involved in rare diseases. These elements are necessary in order to achieve all the goals of the project and could be used for projects about prevention, diagnosis and treatment in a complex and sensitive area like rare diseases.

P. FOLIC ACID EXCESS: HEALTH RISKS EVIDENCE?

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Recent concerns for possible adverse effects due to high Folic Acid (FA) intake brought to a reconsideration of the general FA fortification in food. Such risks, relating to vitamin B12 metabolism interferences and to cancer promotion, should be characterized from a qualitative and quantitative point of view. Herewith we discuss the preliminary results of a work presented to the European Food Safety Authority; we evaluated the available evidences on the correlation between biomarkers of folate status (folate levels in serum, plasma or red blood cells) and adverse effects of FA in populations in which generalized FA flour fortification has been performed. The following indications emerge:

- internal exposure data, beyond being limited, are not easily comparable because different biomarkers are used as well as units of measurement (eg., ng/ml or nmol/l);
- a narrow population fraction (<5%) over 60 shows a correlation between vitamin B12 low levels and high folates levels;
- for colorectal cancer there is mainly an indirect evidence of protective effect of low folate levels;
- a recent study suggests a correlation between folate high levels and an increased risk for mammary cancer (mainly postmenopausal);
- prostate cancer studies show a risk associated with high vitamin B12 levels, but not with folates;
- there no indications of a protective role towards colorectal, breast or prostate tumours by folate high levels (*i.e.*, the higher quintile or quartile of the selected biomarkers);
- in general, data shows how FA effects should be considered also in relation with B12, and possibly other micronutrients as well.

Folate levels in serum or in plasma associated with an initial increment of adverse effects in vulnerable subjects (middle aged or elderly) are in the ≥25 nmol/l range. Since such levels are reachable in populations in which general food fortifications is being performed, a precautionary approach is recommended toward such procedure, at least in anticipation of further studies. On the other hand, in order to prevent neural tube defects it is advisable to effectively support strategies such as the promotion of a more proper nutrition and the periconceptional supplementation.

The present paper is performed within the ISS-NIH Project "Neural tube defects and folic acid"- Working Unit "Risk-to-Benefit Analysis".

P. TOWARD THE ESTABLISHMENT OF A CHEMICALLY-INDUCED MOUSE MODEL OF HEPATOBLASTOMA

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Hepatoblastoma (HB), the major primary liver malignancy affecting children, especially below 2 years of age, is a very rare, sporadic cancer that is associated to some risk factors such as: low birth weight (and the currently increased survival of pre-term neonates), male gender, and some rare genetic diseases affecting the cell pathways regulating proliferation/differentiation (Beckwith-Wiedemann syndrome, Familial Adenomatous Polyposis, Glucose-Storage Diseases).

Exposure to di(2-ethylhexyl)phthalate (DEHP) has been recently suggested as a factor that may promote HB pathogenesis. DEHP, a plasticizer used to soften PVC, may interact with several nuclear receptors (PPARs, PXR); besides being a widespread pollutant, significant exposure to DEHP may occur through medical devices used in maternal intensive care units, mainly for parenteral nutrition of pre-term neonates.

The main HB molecular marker appear to be the mislocalization of β-catenin, an event occurring in almost all HB cases and leading to uncontrolled cell proliferation within hepatocytes. Despite this, genetic mutations within the β-catenin gene has been shown only in about 40% HB cases.

HB may occur, albeit at low incidence, in mice: mouse HB is especially observed in aged (≥1 year) animals upon chronic exposure in early adulthood to certain chemicals (i.e. benzofuran/BF), thus increasing HB incidence.

As in humans, chemically-induced HB shows a higher incidence in male mice. However, at present, no data do exist on the induction of HB-like alterations in rodents at early life stages and/or upon intrauterine exposure.

The preliminary results of a chemically-induced HB mouse model based on the exposure to DEHP are shown. A comparison with BF exposure has been also performed. CD1 mice have been treated with DEHP (25 and 100 mg/kg body weight per day, corresponding to rodent LOEL of endocrine and liver effects, respectively) and BF (120 mg/kg body weight per day) during pregnancy and up to weaning. The treatment period covered the full critical window for liver development and differentiation on mice, starting at Gestational Day (GD) 12 and completed at birth (GD21). Animals were sampled at weaning (post-natal day/PND 21) and pre-pubertal (PND35) stages.

Preliminary findings indicate that DEHP and BF have similar effects on liver upon developmental exposure, although DEHP-induced effects are more pronounced. Such effects include altered fatty acid and glucose metabolism as evidenced by hepatosteatosis and reduced glycogen storage, as well as by a trend toward intracellular dislocation of β-catenin. Interestingly, DEHP-exposed mice showed overall signs of intrauterine growth retardation.

This work is performed within the frame of the project "Tackling rare diseases yet lacking diagnosis and/or prognosis: a pilot project integrating data collection and experimental studies" supported by a NIH-ISS 2007-2009 grant.

CALLOSAL AGENESIS: A BRAIN MALFORMATION WITH POLYGENIC ORIGIN. IDENTIFICATION OF CANDIDATE GENES AND *LOCI* THROUGH A MULTIDISCIPLINARY APPROACH OF CLINICAL, CYTOGENETIC AND MOLECULAR STUDIES OF A LARGE SET OF PATIENT WITH *CORPUS CALLOSUM* ANOMALIES

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Introduction. Callosal agenesis (ACC) can manifest as partial or complete; moreover ACC may occur as an isolated malformation or as a component of more complex malformation syndrome.

Several known genes and molecules cause defects in murine callosal development. So, it's reasonable to hypothesize that, also in human, a large number of genes with evolutionary conserved functions might be involved in callosal development.

Some instruments help research about candidate genes for CC formation and agenesis in humans. For of all, cytogenetic abnormalities and chromosomal breakpoints in ACC patients are useful to select chromosome *loci* with candidate genes (positional candidates). Also ACC animal models are useful to characterize functional genes. Moreover, Array-CGH technique allows a more detailed genome investigation about chromosomal imbalances.

In 2006 we reported a clinical and genetic study regarding 63 ACC patients referred to our Institute. High-resolution karyotype and FISH using subtelomeric probes identified respectively 7 and 3 chromosomal rearrangements (involving *loci* 8p23, 4p15, 10p,10q11, 21 trisomy and *loci* 1p36, 1q44, 6q27, 13q32).

Aims and Methods. To expand the previous collected cohort extending the analysis on new patients with any type of ACC referring to our Institute. All them will undergo a complete clinical, neuropsychiatric and dysmorphological evaluation.

To perform cytogenetic analysis (high-resolution karyotype and FISH analysis using subtelomeric probes in all new collected subjects; array-CGH analysis in selected patients) in order to detect new chromosomal rearrangements.

To perform molecular analysis of ACC candidate genes selected on the basis of chromosomal location (detected in chromosomal rearrangements found both in our patients and in the literature) or by functional homology data with mouse ACC genes.

Results. We collected 48 subjects with complete ACC (10 isolated ACC and 38 not-isolated ACC) and 54 subjects with partial ACC (11 isolated ACC and 43 not-isolated ACC).

High-resolution karyotype detected 12 chromosomal imbalances, while subtelomeric analysis detected 5 imbalances.

No new array-CGH imbalance has been detected until now.

No mutation has been detected in the following genes, until now: AKT3, NRP1, Netrin1 (performed in 25 subjects), ARX (20 subjects), HESX1 (1 subject) and EMX1 (3 subjects).

Conclusions. On the basis of our and other authors' studies we can reasonably conclude that AKT3 gene is not involved in callosal agenesis. Other investigation are needed to understand the role of Netrin1, NRP1, HESX1 and EMX1.

PROGNOSTIC AND PREDICTIVE MARKERS IN THYMIC EPITHELIAL TUMOURS (TET): A TISSUE MICROARRAY (TMA) - BASED MULTICENTER STUDY

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Background. Thymic Epithelial Tumors (TET) constitute both a diagnostic and a therapeutical challenge, as the traditional clinical staging criteria and the recently developed diagnostic pathological criteria although relevant do not disclose the biological tumor potential. Usually benign, some TET behave as malignant tumors, and relapse several times and/or develop multiple intrathoracic metastases. The rarity of TET prevented from establishing an effective therapy for malignant cases. The identification at the diagnosis of tissue-based statistically significant prognostic and predictive factors could allow to set new therapeutical strategies.

Material and Methods. 210 TET cases occurring between 1996 and 2008 were collected in different Institutions from the North, Center and South of Italy, based on a long-lasting collaboration among Pathologists and Clinicians. Paraffin-embedded duplicate sample cylinders of the tumors, 2 mm in diameter, were punched and included in a multitumor Tissue Micro Array (TMA). Normal tissues (normal thymus, hyperplastic thymuses and thymi adjacent to tumors) were also arrayed in TMAs. ErbB family of thyrosine kinase receptors, cell cycle regulatory protein (p16/p21/p27/Cyclin D1), apoptotic and antiapoptotic factors (bax, p53/bcl2), marker of proliferation such as KI67, angiogenic factor such as Vascular Endothelial Growth Factor (VEGF), VEGF receptors (R1, R2, R3), adhesion/signalling molecules such as E-Cadherin, b-Catenin, Trop-1/Ep-CAM expression was investigated by immunohistochemistry and related to the available clinical and follow-up data.

Results. The 2004 WHO TET tumor classification was applied; TMA staining data analysis is in progress. Among the first results obtained, we found that in the ErbB family receptor, HER2/neu was very rarely and heterogeneously expressed in thymoma/thymic carcinoma, in cytoplasmic or membranous localization. Along the entire spectrum of TET, EGFR expression was seen, its hyperexpression being positively correlated with the increased epithelial cell density occurring along the B1<B2<B3 sequence. VEGF-R2 was variably expressed in the thymi examined. In most tumors VEGF-R2 was found to be variably and heterogeneously cytoplasmically-located, the membranous expression being observed only in a tumor subset. Trop-1/Ep-CAM staining was observed cytoplasmically located along the entire spectrum of TET, and strongly expressed on the cell membrane in some cases.

Conclusions. From these very preliminary data, in addition to EGF-R, we have identified two possible targets of therapeutic interventions: Trop-1/Ep-CAM (humanized antibodies) and VEGF-R2 (small TK-inhibitors). The TMA-based approach could constitute a useful tool to identify in TET "a molecular signature" with significant prognostic/predictive value.

A MULTIDISCIPLINARY APPROACH FOR THE INVESTIGATION OF HYPERPARATHYROIDISM-JAW TUMOUR SYNDROME

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HRPT2 germline mutations are responsible for more than half of cases of Hyperparathyroidism-Jaw Tumor syndrome (HPT-JT) and for a subset of Familial Isolated Hyperparathyroidism (FIHP). In addition, HRPT2 mutations have been identified also in sporadic parathyroid carcinomas (67-100%) and, very rarely, in adenomas (0-4%). The protein encoded by HRPT2 is named parafibromin, and its expression is impaired in parathyroid tumors from subjects affected by HPT-JT and in sporadic parathyroid carcinomas.

The first part of our project focused on a clinical, genetic, and histopathologic study in three unrelated Italian kindreds with HPT-JT and FIHP. HPT-JT and FIHP patients had similar laboratory, clinical, and demographic features and shared primary hyperparathyroidism and other neoplasms, the most common of which was uterine polyposis.

The kindreds had also the same genetic background, characterized by the occurrence of inactivating mutations of the HRPT2 gene: in fact, we identified, by direct sequencing of the entire coding region and the intron-exon boundaries of the HRPT2 gene, germline mutations in the probands and in all affected patients of the three kindreds.

Genetic analysis of tumor samples demonstrated a second somatic HRPT2 mutation only in a parathyroid adenoma and no cases with loss of the wild-type allele or methylation of the HRPT2 promoter, even though immunohistochemical analysis demonstrated loss of nuclear parafibromin expression in all tumors, including a uterine polyp.

Our study also demonstrated hyperparathyroidism in FIHP patients with HRPT2 mutations is often characterized by single-gland involvement, and in most cases a limited parathyroidectomy achieved a long-term cure.

Moreover, we are also collecting a large series of sporadic parathyroid tumors, comprising to date 45 adenomas and 3 carcinomas. We performed direct DNA sequencing of the complete HRPT2 coding region in the three carcinomas and we detected a somatic heterozygous nonsense mutations only in a case of parathyroid carcinoma occurring in a young woman that previously underwent surgery for the excision of a jaw tumor. We also demonstrated the absence of nuclear anti-parafibromin immunoreactivity in the parathyroid tumor, but intense immunostaining was present in the jaw tumor.

Finally, we are currently analyzing parafibromin expression by western blotting in our series of sporadic parathyroid tumors; our preliminary results indicate that there are no substantial differences in parafibromin expression among different parathyroid adenomas; a correlation with HRPT2 mRNA expression and parafibomin immunohistochemistry is in progress.

USEFULNESS OF MLPA IN THE MOLECULAR DIAGNOSIS OF LISSENCEPHAY AND NEURONAL MIGRATION DISORDERS

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Lissencephaly (LIS), pachygyria and Subcortical Band Heterotopia (SBH) represent a malformative spectrum of abnormal neuronal migration, often resulting from mutations of either LIS1 or DCX genes. Most children have severe developmental delay and infantile spasms, but milder phenotypes are on record, including posterior SBH owing to mosaic mutations of LIS1. LIS1 mutations result in more severe LIS in the posterior brain regions (p>a gradient). LIS1 is involved in both Isolated Lissencephaly Sequence (ILS) and Miller-Dieker Syndrome (MDS). ILS is caused by intragenic mutations or by internal deletions of LIS1 whereas MDS is caused by deletions of contiguous genes in 17p13.3, including LIS1. DCX mutations usually cause anteriorly predominant LIS (a>p gradient) in males and SBH in females. Mutations of DCX have also been found in males with anterior SBH and in female relatives with normal brain magnetic resonance imaging.

We selected two distinct groups of patients. The first group of patients showed p>a LIS not including MDS; in all these patients FISH for the 17p13.3 region gave negative results. The second group of patients showed sporadic, diffuse, or anteriorly predominant SBH. We initially performed DNA sequencing of LIS1 in patients with p>a LIS and DNA sequencing of DCX in patients with SBH. Subsequently, we performed Multi Length Probe Amplification (MLPA) in those patients who were mutation negative.

In patients with p>a LIS, MLPA identified small genomic deletions/duplications of LIS1 in about 82% (19/25) of patients who had previously been tested unsuccessfully with both FISH and DNA sequencing. Overall, small genomic deletions/duplications, represented 49% (19/39) of genomic alterations and brought to 87% (39/45) the number of patients in our series in whom any involvement of LIS1 could be demonstrated. In order to characterize the breakpoint regions, we performed Long Range PCR in five patients with deletions. We demonstrated that, in four of them, deletions were caused by Alu elements mediated recombination, suggesting that LIS1 is particularly prone to undergo recombination between Alu elements. In 3 of 11 women (27%) with diffuse, or anteriorly predominant SBH, MLPA uncovered two deletions encompassing exons 3 to 5, and one involving exon 6, bringing the percentage of DCX alterations from 52% to 65% in our series. SQF-PCR and Southern blot analysis confirmed the deletions. MLPA should be used in the molecular diagnosis for p>a LIS and diffuse, or anteriorly predominant SBH.

DIAGNOSTIC AND THERAPEUTIC TARGET OF SYSTEMIC AMYLOIDOSIS: VALIDATION OF NEW DIAGNOSTIC TOOLS AND DEVELOPMENT OF NEW DISEASE MODELS

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New diagnostic and therapeutic strategies for systemic amyloidoses can be based on detailed information on composition of natural fibrils and structural/functional characterisation of tissue-specific molecular interactors locally active on amyloid deposition. We have developed a proteomic approach in order to identify the pathogenic protein in the biopsy of the abdominal fat, which is the tissue of choice for the diagnosis of systemic amyloidosis. This represents a novel powerful diagnostic tool for assessing unequivocally the type of amyloidosis.

We are also investigating the physiologic proteomic pattern of subcutaneous adipose tissue. The definition of proteins expressed in non-pathological tissue allows identifying enzymes and proteins that play key roles in metabolic pathways potentially affected by the amyloid deposition.

Results provided by the proteomic approach have been exploited in the assessment of new methods of fibrillogenesis of globular proteins that mimic the natural environment. In particular through the combination of collagen, collagen plus heparin and minimal amount of truncated β2-microglobulin we can now grow amyloid fibrils at neutral pH.

In particular, we have demonstrated that homogenous heparin at concentration of 1-3 μg/ml, consistent with concentration occurring *in vivo* during haemodialysis, strongly favours amyloid formation.

Our data suggest that heparin would favour fibrillogenesis by catalyzing the generation of soluble oligomers correctly oriented in a cross-beta structure.

The mechanism of early aggregation is under extensive investigation and we have discovered that certain strands of β2-microglobulin have a prominent role in the early phases of oligomerization.

The multiple methods of fibrillogenesis, whose dynamics can be now monitored at the molecular level, allow investigating the properties of new and old drugs able to prevent protein fibrillogenesis.

Extremely promising results have been obtained through a collaborative work with Mario Negri Institute in Milan which is providing new analogues of tetracyclines and other heterocyclic small molecules that *in vitro* display significant anti-amyloidogenic property. Furthermore, we have investigated the development of amyloid deposits, constituted by the lipoprotein ApoA-II, in the aging CD1 mouse strain.

We have found that the amyloid is deposited in spleen and liver, but also in the heart, an organ that is rarely involved in other amyloid mice models. The amyloid deposition in the heart is now under extensive investigation in order to correlate the molecular abnormalities and the histo-pathological features with the echocardiographic and MRI patterns.

GENETIC ABNORMALITIES OF COMPLEMENT MOLECULES IN HEMOLYTIC UREMIC SYNDROME

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Hemolytic Uremic Syndrome (HUS) is a thrombotic microangiopathy with manifestations of hemolytic anemia, thrombocytopenia and renal impairment. Genetic studies have shown that mutations in CFH, MCP and CFI predispose to atypical-HUS (aHUS).

C3 plays a central role in the activation of both classical and Alternative Pathways (AP) of the complement system. Complement Factor B (CFB) is cleaved by CFD yielding the noncatalytic chain Ba and the catalytic subunit Bb, which associates with C3b to form the AP C3 convertase. Mutations in CFB were reported in 2 patients from the Spanish cohort and a preliminary report described 18 C3 mutations in patients from the Paris and Newcastle cohorts.

To evaluate the frequency of CFB and C3 mutations in our cohort we selected 43 patients, based upon the following criteria: no mutations in CFH, MCP and CFI, C3 serum levels below normal range (n.r. 83-177 mg/dL), and C4 serum levels within normal range or elevated (n.r. 15-45 mg/dL). We screened also 46 to 96 healthy unrelated controls.

In C3, 4 mutations were found in 3 patients determining R570W, S1041R, I1135T and T1361N aminoacidic substitutions. These mutations were not found in 46 healthy controls. Functional data are not available yet, however R570 is located very close to the C3aReceptor binding site and the three other mutations are located within the alpha-chain of C3b fragment. In particular, I1135 is located in a strain of 5 CR2 interacting residues.

In CFB gene, one heterozygous mutation was found, determining a R138W aminoacidic change. This mutation has not been previously described and it has not been found in 96 healthy controls. Moreover 8 polymorphic variants were identified in CFB gene (6 have been previously reported, namely, T26A in exon 1, C94T and G95A in exon 2, G450A in exon 3, G754A in exon 5, and A1693G in exon 13, while two are new, namely C672T in exon 5 and T1137C in exon 8).

Comparison of C94T frequency in the patients and in the controls showed a statistically significant association of the C94T polymorphism with aHUS (genotypic distribution $p=0.022$, allelic distribution $p=0.006$). The allelic distribution of the G450A is also statistically significant, showing a higher frequency of the rare allele (A) in the control population as compared to the patients ($p=0.016$) thus suggesting a possible protective role.

In conclusion our findings support previously reported data indicating C3 and CFB involvement in aHUS.

P. EVALUATION OF GENETIC AND ENVIRONMENTAL FACTORS IN A COHORT OF TWINS WITH AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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We started an Italian nation-wide epidemiological study on twins affected with Amyotrophic Lateral Sclerosis (ALS) to estimate: i) disease concordance in monozygotic and dizygotic twins; ii) genetic (heritability) and environmental variance components of susceptibility to ALS; iii) in concordant pairs, if any, recurrence risk ratio, disease discordance time and second-twin progression rates to the disease.

Possible twins are being identified linking the Italian Twin Registry to ALS patients' lists provided by 3 ALS regional Registries and 14 diagnosis Reference Centres.

So far, among 4982 patients (60% males), diagnosed with "definite", "probable" or "possible" ALS since 1990, we ascertained 29 twins (none of them belong to the same pair) plus 18 patients whose twin status is under ascertainment.

Twenty-two ALS twins (15 males) are from same sex pairs and 7 (6 male) belong to unlike sex pairs. As regards living status, in 1 pair both twins (ALS twin and co-twin) are deceased, while in 15 pairs 14 ALS twins and 1 co-twin are deceased. Health status or cause of death of co-twins will be ascertained by a neurologist.

Life exposure to putative ALS risk factors will be investigated through a co-twin control study. Collection of biological material from ALS and unaffected twins is also envisaged.

CHARACTERIZATION OF THE MOLECULAR AND CELLULAR MECHANISMS UNDERLYING THE LIVER PATHOGENESIS IN HEMOPHAGOCYTIC SYNDROMES (HS)

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Hemophagocytic Syndrome (HS) is a severe and often fatal syndrome resulting from potent and uncontrolled activation and proliferation of T lymphocytes, leading to excessive macrophage activation and multiple deleterious effects. This project is devoted to a better characterization of the mechanisms underlying the liver pathology of the HS. Our preliminary results indicate at least two new mechanisms potentially involved in HS progression.

We have characterized the role of chemerin, the ligand of the chemotactic receptor ChemR23, in inducing the recruitment and co-localization of Myeloid Dendritic Cells (M-DC), Plasmacytoid DC (P-DC) and NK cells. The three cell types express high levels of functional ChemR23 and chemerin is highly expressed in the liver. Chemerin expression was not found in these leukocyte populations in none of the conditions investigated. To gain insight in the cell type responsible for chemerin production in the liver we decided to use in situ hybridisation. To this goal a specific probe was cloned in pBSKS+ vector and sections from human liver and lymph nodes, these experiments are still ongoing. Finally, chemerin did not stimulate NK cell *in vitro*, cytokine production, NK cell degranulation and killing of K562 cells. We propose that chemerin, in addition to certain chemokines known to be produced by activated Kupffer cells, such as CCL20, may induce the colocalization of innate immunity effector cells in pathological conditions, such as HS.

9 patients with clinical manifestations resembling HLH were classified according to current diagnostic criteria of Hystiocyte Societ. Patients presented the following clinical manifestations were as follows: fever was observed in 100% of patients, splenomegaly 100% of subjects, single lineage cytopenia (anemia, thrombocytopenia or neutropenia) 88%, hemophagocytosis (78%), Impaired NK cell cytotoxicity (100%).

Genetic analysis of PRF1 and UNC13D was performed in all subjects. Three of them displayed mutations of PRF1, while another one carried UNC13D mutation. We have analyzed NK and NK-T cells in two patients affected by Hermansky-Pudlak type 2. Preliminary data suggest abnormal distribution of NK cell subset and complete absence of NK-T cells. In these subjects, analysis of CD63 expression on cell surface has shown increased levels on cell surface. Moreover, CD63 expression is not regulated after activation, suggesting that CD63 transport to plasmamembrane is altered in these subjects.

P. ALTERNATIVE SPLICING, CAN BE A MARKER OF UVEAL MELANOMA

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Cancer cells can alter their adjacent stroma to form a permissive and supportive environment for tumor progression and produce a range of growth factors and proteases that modify their stromal environment. These factors disrupt normal tissue homeostasis and act in a paracrine manner to induce angiogenesis, inflammation, as well as activation of surrounding stromal cell types.

The induction of inflammation in the tumour stroma also results in production of a range of factors including Extra Cellular Matrix (ECM) components that promote tumour progression. Periostin is a secretory omodimeric protein of the ECM with an apparent molecular mass of 180 kDa, involved in cell adhesion and tumor formation that is produced in the stromal environment.

Recently, it has been reported that the periostin expression levels increase in primary and metastatic melanoma due to both stromal and tumoral cells production. Although splicing variants of periostin have been described in bladder cancer tissues, to date nothing is known on periostin isoforms expression in melanoma.

To study periostin isoforms as new potential tumoral markers, we analyzed the alternative splicing of the human periostin transcript in tumoral cells and fibroblasts isolated from cutaneous metastatic melanoma tissues. Preliminary results indicate that both types of tumor-derived cells produce periostin mRNA, but only melanoma cells express exon 17 in 50% of cases (3/6).

On the contrary, all (4/4) the uveal melanoma tissues analyzed so far express isoforms of periostin containing the exon 17, thus suggesting that exon 17 of human periostin can be a potential marker of this tumor.

QUALITY OF LIFE AND DISABILITY IN FABRY DISEASE

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Introduction. Fabry Disease (FD) is an X-linked lysosomal storage disorder (prevalence about 1 : 100 000) caused by a genetic defect associated with a lack of alpha-galactosidase A enzyme activity. This mutation causes insufficient breakdown of lipids, which build up to harmful levels in the eyes, kidneys, autonomic nervous system, and cardiovascular system with a clinical systematic involvement of various organs. FD patients often complain of muscular pain, fatigability and asthenia referring them as one of the major causes of deterioration of Quality of Life (QoL) and disability: these symptoms may involve deambulation and motor performance of superior limbs. To clarify the possible causes of referred symptoms in FD we presented a project in the context of the Call for Rare Disease. The study is still ongoing, here we present data about QoL and disability.

Methods. We evaluated QoL and disability by the following measures: Disability Arm Shoulder Hand questionnaire (DASH), the Lumbar Spine outcome assessment instrument (NASS) and Short Form-36 (SF-36). We studied 5 patients affected by FD (male/female: 4/1, mean age and SD: 37±16, range: 23-62).

Results. Concerning the QoL subscores the comparison between FD patients and normal value showed a statistical significance about the bodily pain, general health and vitality domains (respectively $p<0.001$; $p<0.001$ and $p=0.02$). Concerning the other used questionnaires we found a statistical significance about the domain of NASS regarding pain-disability ($p=0.008$).

Conclusions. The obtained results, in accordance with the literature and our clinical experience, showed a higher tendency of FD patients to complain pain respect to the Italian norms and this tendency probably results in a worst general health. Although this topic has been investigated, pain is not studied in a comprehensive way. To assess the aetiology of pain we will add to our protocol pain specific questionnaires and we will related these results to the Near Infrared Spectroscopy in order to acquire data on the oxygenation of muscles and deregulation of microcirculation.

TARGETING THE PROGNOSTIC AND METASTASIS-PREDICTING SURFACE PROTEOGLYCAN FOR IMMUNOTHERAPEUTIC TREATMENT OF SELECTED SARCOMAS

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A recent analysis of the transcriptional and protein expression profile in primite and metastatic lesions of more than 70 soft-tissue sarcoma patients reveals an unprecedented role for the cell surface proteoglycan NG2 in predicting with more than 50% probability future, post-operative metastatic disease.

This finding, taken together with the lower survival rates of patients with enhanced NG2 expression, assigns to the proteoglycan a value as independent prognostic factor and has incited a multicenter extension of the investigations, with particular emphasis on relapsing frequences and therapeutic responses. In parallel efforts, by employing more than 30 sarcoma lines established from surgical specimens, *in vitro* growth, adhesion and cell migration assays and transplantation into wild type and transgenic mice, we have resolved some of the cellular and molecular mechanisms underlying the modes through NG2 may promote tumour growth and metastasis formation. DNA microarray-based gene profiling and combined proteomic and phosho-proteomic profiling was employed to delineate the gene and signalling networks associated with the NG2 pro-tumorigenic role. The acquisition of this background information has provided a significant support for the immunotherapeutic targeting of the proteoglycan in soft-tissue sarcoma patients, based upo the exploitation of anti-NG2 anti-idiotypic approaches (and agents) recently proven to be effective on advanced melanoma patients.

To this end we have specifcally examined some of the immunological traits of antibody MK23-3 found to be able to induce T cell responses cross-reacting with NG2 and discovered to behave as an "heteroclitic vaccine". Combined bioinformatic analyses and *in vitro* assays on T lymphocytes suggest that this ability of anti-idiotypic antibody to induce the observed immune responses may be explained, at least in part, by the homology of variable heavy chain of this antibody with short 10-17 amino acid stretches of NG2.

These elaborations have set the ground for also identify particularly immunogenic peptide sequences that could be used in conjunction with anti-idiotypic antibodies, also in non-responders.

In a separate experimental series aimed at generating immunological therapeutic reagents to be trasfereed to clinical settings on soft-tissue sarcoma patients we have produced a panel of 63 anti-NG2 monoclonal antibodies. A first characterization of these antibodies have yielded unexpected variations in the ability of the antibodies to recognize the antigen on diverse soft-tissue sarcoma cells suggesting a structural-functional diversity of the proteoglycan.

Functional analyses are in progress to address the tumorigenic significance of these variations and potential utility of these reagents as tumour abrogating agents *in vivo*.

P. EVIDENCES FOR ASSOCIATION OF THE CASP8-652 6N DEL PROMOTER POLYMORPHISM WITH AGE AT DIAGNOSIS IN FAMILIAL BREAST CANCER CASES

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Introduction. Recently, a study conducted in Chinese individuals reported that a 6-nucleotide deletion in the promoter of the CASP8 gene (the -652 6N del; rs3834129) was strongly associated with decreased cancer risk in several types of cancers including Breast Cancer (BC).

Materials and methods. We studied the effect of this polymorphism in a series of 580 Italian BC index cases and 406 controls. The cases were originally ascertained as eligible for mutation analysis in BRCA genes. All the females affected with BC as the first diagnosed cancer, who tested negative for BRCA disease-causing mutations, were included in this study. The controls were Italian female blood donors who were ≥45 years at the blood draw. The genotyping was performed comparing the length of PCR fragments of the normal and the deleted allele with a size marker using Denaturing High-Performance Liquid Chromatography.

Results. In cases and controls, common homozygous (nor/nor), heterozygous (nor/del) and rare homozygous (del/del) were 162 (27.9%), 301 (51.9%) and 117 (20.2%); and 106 (26.1%), 206 (50.7%) and 94 (23.2%), respectively. By logistic regression analysis adjusted for age, we observed that the *Odds Ratio* (OR) for heterozygous and rare homozygous compared with common homozygous was 1.05 (95% Confidence Interval (CI)=0.74-1.49) and 1.09 (95% CI=0.71-1.67), respectively, and the per-allele OR was 0.96 (95% CI=0.78-1.18). These results provided no evidence for association between the -652 6N del polymorphism and familial BC unlinked to BRCA genes. We performed additional

analyses and investigated the association between the polymorphism and age at BC diagnosis in cases. The three genotypes were collapsed into two: (del/del), and (nor/-) by aggregating common homozygous and heterozygous cases; the age at diagnosis was categorized into four classes according to the pertinent age centiles (25^{th}: 35; 50^{th}: 43; 75^{th}: 50). We assessed the associations between each age class and genotype by the logistic regression model and observed a statistically significant association between these two variables. In particular, our results suggested an increasing trend of the del/del genotype with later age at BC diagnosis (trend test p-value=0.01).

Conclusion. We observed that the -652 6N del polymorphism of CASP8 was associated with age at diagnosis in familial BC cases. Thus, we suggest that this polymorphism may have an effect in postponing the BC onset in predisposed individuals.

ROLE OF THE DYSTROPHIN-ASSOCIATED GLYCOPROTEIN COMPLEX IN LIMB-GIRDLE AND CONGENITAL MUSCULAR DYSTROPHIES: FROM MOLECULAR PATHOPHYSIOLOGY TO POTENTIAL THERAPY (7DR1)

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The aim of our project, which involves four groups, is to improve our understanding of the underlying mechanisms that produce a dystrophic phenotype, focusing our studies on the components of the Dystrophin-associated Glycoprotein Complex (DGC), that is dystrophin, the dystroglycans (α and β), the sarcoglycans (α, β, γ and δ), sarcospan, the syntrophins (α1, β1and β2) and α-dystrobrevin. We focused our studies on: i) the analysis of muscle tissue gene expression profile in cases of Congenital Muscular Dystrophies (CMD) due to merosin deficiency or associated with α-DG hypoglycosylation; ii) the role of dystroglycan in cell adhesion and cell signalling; iii) the emerging functions of dystrobrevin as motor adaptor and signalling scaffold protein.

We (UO2, Ricci-Brancaccio) have analyzed the dystroglycan α/β interface, in order to better characterise the reciprocal intersubunit binding sites. We have identified four amino acids that are crucial for the interaction between the two subunits and found that their substitution with Ala residues in transfected Ebna-293 cells altered the DG processing, preventing the cleavage that separates α-DG and β-DG. A group of patients affected by different types of muscular dystrophies have been analysed in order to assess the possible contribution of the DG α/β interface to the pathology of skeletal muscle. In addition, characterization of muscle biopsies of patients with a known diagnosis of CMD has been performed using oligonucleotide microarrays technology, genetic analysis and immunohistochemistry Cognitive impairment and mental retardation are often associated with muscle atrophy in some congenital forms of muscular dystrophies, as the laminin α2-deficient CMDs.

We (UO1, Petrucci) used the C57BL dy^{2J}/dy^{2J} mouse, an animal model of α2-deficient CMD, and littermate controls, to analyse by western blot and immunohistochemistry the level of DGC components both in skeletal muscle and brain extracts. We found that dystroglycan processing was altered in both tissues, and syntrophin was upregulated in skeletal muscle of dy^{2J}/dy^{2J} compared to control mice. The cytoplamic components of the DGC, syntrophin and dystrobrevin are known to be involved in signal transduction and DGC stabilization. We (UO4, Ceccarini) have characterized the interaction between β-dystrobrevin and the molecular motor kinesin, and hypothesized that this interaction could be functional to the

intracellular transport of dystrobrevin and its binding partners, including PKA regulatory subunits and dysbindin, a component of the Biogenesis of Lysosome-related Organelles Complex 1 (BLOC-1) that regulates synthesis and trafficking of lysosome-related organelle. We found that both PKA-dependent phosphorylation of dystrobrevin and the presence of Ca^{++} can significantly lower the binding affinity of dystrobrevin-kinesin interaction, whereas dystrobrevin-dysbindin interaction was unaffected.

Among the new dystrobrevin interacting protein we have identified (UO3, Macioce) iBRAF/HMG20a, a member of the HMG-proteins that modulate chromatin structure. We have characterized β-dystrobrevin interaction with iBRAF by *in vitro* and *in vivo* assays, and obtained results that suggest β-dystrobrevin, through its association with iBRAF, may be involved in regulating chromatin dynamics and consequently play a role in the activation of neuronal specific genes.

ESTABLISHMENT OF A EUROPEAN NETWORK OF RARE BLEEDING DISORDERS (RBDS)

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The aim of the project is to set up a network of Italian and other European Centres dealing with patients affected by RBDs in order to implement the already existing RBDD database (www.rbdd.org).

The main partner, at present the only Italian participating centre, involved other European Centres in the testing phase of the database meant to optimize its on-line functions, verifying the proper setting of the help, controls on guided choices, clarity of field-browsing, simplicity of interrogation tools. Partners were able to access the database through activation of an account and a protected certification. After an initial data input, partners cited anomalies, logical, browsing and comprehension errors, possible improvements or modification proposals. The changes considered fundamental for the correct working of the database will be made, but only after an agreement has been reached within the network.

Moreover, Associazione Italiana Centri Emofilia (AICE), a reliable association regarding treatment Centre coordination and patients assistance in the field of hemophilia, was involved to create an Italian National Registry on RBDs, similar to that created by us at European level. This collaboration was aimed to link the information on RBDs patients inserted in the Emocard, the AICE computer-based clinical data collection system, with those inserted in the European and RBDD databases. To understand whether the two databases could communicate and collect data homogenously, a comparison of the respective fields was carried out, showing that the two databases are similar, because the majority of information requested by RBDD are already contained in Emocard. However, few important fields (*e.g.* parents' consanguinity, antigen level, bleeding score) present in the RBDD questionnaire are not foreseen in Emocard. To uniform the two data collection forms, additional pages will be added to the Emocard questionnaire. Emocard information on RBDs patients will be periodically extracted and sent to Flora Pevyandi, responsible of the European project, who will try to improve the harmonization of data collection, analysis and extraction of those data which will be necessary to draw therapeutic guidelines. This will lead us to avoid duplication of data insertion.

In conclusion, it is desirable that within next year, all Italian Centres affiliated to AICE will adhere to this project with the aim of creating a National Registry, not yet available. Data collected in the Italian cohort will implement the already large European cohort, thus allowing to draw adequate statistical analysis, useful to clinical practice, by organization of therapeutic guidelines.

BIOCHEMICAL AND CELLULAR REAL-TIME BIOMARKERS OF DIAGNOSTIC AND PROGNOSTIC VALUE IN THE MANAGEMENT OF KAWASAKI'S DISEASE

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Two different points have been analyzed as valuable biomarkers of diagnosis and progression in blood samples from patients with Kawasaki Disease (KD): oxidative stress and blood cell integrity and function.

Background. Persistent arterial dysfunction in patients with a history of KD and an integral role of oxidative stress in the development of cardiovascular disease are increasingly recognized. We sought to test the hypothesis that oxidative stress is increased in KD patients by evaluating different possible plasmatic and cellular biomarkers. In addition, platelets are increased in their number in KD and their alterations have been suggested to exert a pathogenetic role.

Methods. We measured by Electron Paramagnetic Resonance spin trapping with 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpirrolydine (CPH) the production of free radicals in whole blood of KD patients compared to control health subjects.

As concerns blood cells, erythrocytes and platelets were analyzed by static and flow cytometry. Regarding erythrocytes, redox imbalance and expression of proteins (glycophorin A and CD47) involved in cell aging and death were evaluated. Regarding platelets, in order to mimic the clinical situation, an immunologic stimulus has been used (opsonized zymosan) and their aggregation and adhesion features as well as their death susceptibility have been analyzed.

Results. Compared with controls, patients with KD had significantly higher rate of free radical production as demonstrated by the increase (+86% $p<0.001$) of the rate of CP• radical formation. The rate of CP• formation further increased about 4-5 times after addition of the transition-metal chelator EDTA, leading to the hypothesis that the increased radical formation may be mediated by catalytically active Iron. After therapy (aspirin 2mg/Kg/die) the rate of CP• formation was decreased, but still significantly higher than controls (+30% $p<0.001$). As concerns erythrocytes, a decreased expression of glycophorin A and CD47 has been detected in KD patients with respect to healthy donors.

For platelets, main finding deals with their increased aggregability and, more importantly, the increased Phosphatydilserine (PS) externalization without further sign of cell death (*e.g.* no caspase activation).

Conclusions. Our findings suggest: i) oxidative stress is increased in KD patients at the onset of the disease. We suggest the presence of an inflammatory condition perhaps mediated by Iron overload and/or decompartmentalization; ii) the increased platelet counts in these patients could be due to a defective death pathway whereas PS externalization could be associated with an increased vascular risk. Altogether these findings suggest that further studies should be performed in KD blood samples in order to better assess these hypotheses that could help in the clinical management of this disease.

IMPAIRED CORTICOSTRIATAL LTD AND SYNAPTIC DEPOTENTIATION IN A MODEL OF DYT1 DYSTONIA DEPENDS ON DYSREGULATED CHOLINERGIC SIGNALING

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Clinically unaffected DYT1 gene carriers exhibit subtle abnormalities in motor behavior with impaired sequence learning. Corticostriatal synaptic plasticity is believed to play a central role in motor learning. We investigated possible changes in synaptic plasticity in transgenic mice expressing either the human Wild-type TorsinA (hWT) or Mutant TorsinA (hMT). High-Frequency Stimulation (HFS) induced Long-Term Depression (LTD) in MSNs recorded from control and hWT mice, but failed to cause LTD in hMT littermates. Pretreatment with D1 or D2 dopamine receptor agonists was unable to restore LTD, whereas combined application of both agonists partially rescued LTD. Because a paradoxical, excitatory D2-dependent effect has been shown in cholinergic interneurons from hMT mice, we tested the possibility that an excess in Acetylcholine (ACh) striatal levels could impair LTD.

Pre-incubation with either hemicholinium, which depletes endogenous ACh, or with the M1-preferring ACh-muscarinic receptor antagonists, pirenzepine and trihexyphenidyl, restored LTD. In the absence of magnesium, HFS was able to induce a Long-Term Potentiation (LTP) in MSNs recorded from either controls or hWT mice. In hMT mice the magnitude of LTP was significantly higher than in hWT and controls. Once obtained a stable LTP, a Low-Frequency Stimulation (LFS) protocol was able to induce a Synaptic Depotentiation (SD) both in controls and hWT mice.

However, LFS failed to determine SD in hMT mice. Similarly to what observed for LTD, both hemicholinium and pirenzepine rescued SD when applied after LTP induction. Likewise, in mice deficient for the muscarinic autoreceptor M4, we did not observe SD. Together, these results suggest that a dysregulation in the dopaminergic control over cholinergic signaling impairs synaptic plasticity in the striatum of DYT1 transgenic mice. These functional alterations might represent the cellular bases for the motor abnormalities observed in non-manifesting DYT1 carriers.

RELIABILITY AND EFFICACY OF THE CURRENT DIAGNOSTIC APPROACH IN NARCOLEPSY AND SEARCH FOR NEW GENETIC MARKERS

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Introduction. Narcolepsy is a chronic disease characterized by Excessive Daytime Sleepiness (EDS), typically associated to cataplexy and other phenomena due to the abnormal occurrence of REM sleep elements during wakefulness and frequent sleep/wake transitions. Evidences indicate that its pathogenesis is due to a dysfunction in hypothalamic neurons producing hypocretin (also called orexin), a neurotransmitter involved in sleep/wake regulation.

Aims. To obtain a detailed knowledge of the clinical, neurophysiological, and nocturnal aspects of narcolepsy.

Methods. One hundred and fourteen patients (73 males and 41 females, age 42.7±16.9) with narcolepsy/cataplexy were consecutively recruited for this study by four Research Units. The diagnosis of narcolepsy/cataplexy was based on the International Classification of Sleep Disorders (ICSD-2) criteria. All patients recruited were in wash out from possible pharmacological treatment since 4 weeks. All patients underwent to: i) clinical diagnostic assessment: an accurate anamnesis, focused to determine essential and associated features of narcolepsy: excessive daytime sleepiness, cataplexy, hypnagogic hallucinations, sleep paralysis, disturbed nocturnal sleep, automatic behaviours and moreover, the presence of Restless Leg Syndrome (RLS), and Sleep Related Breathing Disorders (SRBD); ii) Anthropometric assessment: weight, height and Body Max Index (BMI); iii) instrumental diagnostic assessment: 48-hour free running continuous polysomnographic recording (PSG) followed by Multiple Sleep Latency Test (MSLT).

Abnormal muscle activity nocturnal index has been monitored (Periodic Limb Movement during Sleep, PLMS) and Apnea/Hypopnea Index (AHI) determined. Moreover, whenever possible, a sample of Cerebral-Spinal Fluid (CSF) was obtained by lumbar puncture in order to dose hypocretin level.

Results. Clinically, the complete narcolepsy symptom tetrad was present in 18.5% of all patients, RLS in 10.5%, SRBD were absent or with a slight entity (AHI 4.6±12.89). CSF-hypocretin levels were pathological (undetectable or <200 pg/ml). Sleep pattern was characterized by longer NREM/REM cycles, longer intervals between REM episodes in comparison with normal population data. The most important sleep abnormality

characterizing narcolepsy was the occurrence of a REM sleep period at sleep onset (SOREMP). Moreover, the night sleep was characterized by reduced sleep efficiency (84.5±32.44%) - due to fragmented sleep for repeated nocturnal awakenings - and by the presence of PLMS (PLM index 14.9±22.5). All patients showed, on average, a significant increase of BMI (28.0±4.69).

Discussion. Our study confirms that narcolepsy is a complex sleep disorder characterized by several clinical daytime symptoms and nocturnal sleep disorders. It also suggests that the sleep/wake cycle dysregulation may be linked to an alteration of the hypothalamic hypocretin's system.

INCIDENCE OF "CHROMOSOMAL PHENOTYPE" IN MENTALLY RETARDED CARRIERS OF PATHOGENIC COPY NUMBER VARIATIONS (CNVS)

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Array CGH assays showed CNVs in patients with mental retardation, resulted normal to routine cytogenetic analysis, in a percentage of 5-25% depending on techniques used and selection patients method. Frequently, the candidates to array-CGH analysis are patients with "chromosomal phenotype".

The aims of our project "Usefulness of 244K array-CGH in the ascertainment of Cryptic Chromosomal Rearrangements in Mental Retardation and Autism (ASD)" are to know: i) the percentage of CCRs among MR and ASD samples; ii) the influence of "chromosomal phenotype" in their ascertainment. Patients with mental retardation were divided in two groups, made up of 50 patients each, one (group 1) with a "chromosomal phenotype" and the other (group 2) without dysmorphic features, all affected by MR, diagnosed according to the DSM-IV-TR criteria.

The patients were included in the appropriate group according to the score reached following the administration of the clinical checklist published by De Vries, in 2001. Each subject reached a score between 0 and 10, depending on the presence or absence of some clinical features such as MR in family history, small birth weight, abnormal postnatal growth, facial dysmorphisms and congenital abnormalities.

We established a total score of 3 as cut-off, separating group 1 (scoring 3 or above) from group 2 (scoring 2 or less).

Until now, we analyzed a total of 79 subjects (39 males and 40 females), 49 from group 1and 30 from group 2. A *de novo* CNVs, validated by MLPA, has been diagnosed in 20 (13 from group 1 and 7 from group 2) out of 79 MR patients.

The preliminary resulting percentages are: a *de novo* pathogenic CNVs is present in 25.3% (20/79) of MR patients, while in 26.5% (13/49) of group 1, and in 23.3% (7/30) of group 2.

These preliminary results confirm that CCRs have an overall prevalence of 25.3% in MR. Incidence of pathogenic CNVs actually was major in patients with a "chromosomal phenotype". However, it should be noted that the sampling in the first group is almost

complete, while further 20 subjects should be tested in the second group, and this could modify the results. Among 100 enrolled patients with Autism 30 subjects have been analyzed having also MR and a "chromosomal phenotype". Three of them (10%) showed a 16p11.2 CNV (two deletions and one duplication).

INVESTIGATION OF GENETIC AND EPIGENETIC MECHANISMS UNDERLYING BECKWITH-WIEDEMANN SYNDROME (BWS) ON A LARGE COHORT OF ITALIAN PATIENTS

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Beckwith-Wiedemann syndrome is a complex overgrowth disorder which manifests with a continuum of clinical signs ranging from patients presenting with at list three main criteria, complete BWS, through a less characterized subset identified as incomplete BWS, to subjects affected only with Isolated Hemihypertrophia (IH). Children affected by BWS show an increased risk of pediatric tumors. The genetic basis of BWS is complex and involves the 11p15 region by genetic and epigenetic alterations within two domains of imprinted growth regulatory genes: IC1 (~2-7% of BWS) associated with H19 and IGF2 (insulin-like growth factor 2) and IC2 associated with KCNQ10T1, which rules the expression of KCNQ and CDKN1C (~ 50%).

We here report on genetic and epigenetic analyses carried on a cohort of patients (WP1 & 2) extended to about 300 BWS families, including 180 with classical phenotype, 60 incomplete and 50 IH. The joined study allowed to develop an exhaustive flow-chart for BWS genetic test, starting with UPD and KvDMR at IC2 scan, then implying H19 (IC1) methylation analysis to end with CDKN1C sequencing and search of rare chromosomal rearrangements. Different tissues should be investigated in cases in whom data from blood are borderline or discordant with the phenotype. To this purpose, we report on two monozygous twins, with opposite phenotype, one affected with BWS and the other healthy both hypomethylated at KvDMR1 (IC2).

DNA from buccal brush of the "unaffected" twin is under study to detect possible tissue mosaicism.The following specific aims have been achieved: i) molecular analysis of familial transmitted microdeletions involving the H19 DMR region which suggested that IC1 hypermethylation is associated to biallelic expression of IGF2 and biallelic silencing of H19 (WP1, WP2, WP4); ii) refined characterization of unreported Italian CDKN1C-positive cases, evidencing in cDNA from blood the presence of the paternal wild type allele, even if at a reduced amount as compared to the aberrant one. The study of genotype-phenotype correlations in patients with this rare defect will be addressed on a larger sample including a UK cohort contributed by prof Weksberg (WP1, WP2, WP4);

iii) methylation analysis of 11 ICRs within a cohort of 149BWS patients, including 81 with maternal hypomethylation of BWS Imprinting Centre 2. Both partial and complete hypomethylation has been demonstrated in these cases, at PLAGL1 and GNAS/NESPAS DMRs suggesting possible postzygotic origin of a mosaic imprinting error.

Lastly prenatal protocol has been approved by WP3, including the following steps: - recruitment of cases referred by first level, ultrasound unit, foetal imaging, definition of the complete foetal clinical picture, genetic counselling session, invasive procedures, foetal karyotype at 400 band resolution, clinical genetics evaluation, clinical pathology protocol, and follow-up after delivery or termination. Investigation of 10 foetuses with evidence of omphalocele plus other anomalies in two.led to disclose the molecular mechanism in three of them, with two showing hypomethylation of KvDMR1 and one carrying a CDKN1C mutation.

P. TWO INTRAGENIC NIPBL DELETIONS AND ONE CONTIGUOUS GENE SYNDROME DETECTED BY MLPA IN CDLS PATIENTS

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Cornelia de Lange Syndrome (CdLS) is a rare, multiple congenital anomaly/mental retardation disease, characterized by facial dysmorphisms, growth deficiency, psychomotor delay and malformations of the upper limbs. About half of CdLS patients are mutated in the NIPBL gene (5p13.2) encoding for a member of the adherin's family, involved in the processes of chromatid cohesion and enhancer-promoter communication. Mutations in the SMC1L1 and SMC3 genes (two subunits of the cohesin complex) responsible for an X-linked and another autosomal form respectively are thought to contribute to up 5% of all CdLS cases.

We screened for NIPBL and SMC1L1 mutations the first Italian cohort of CdLS patients which has been evaluated by the recently approved diagnostic system and a global score. By this analysis besides detecting NIPBL and SMC1L1 mutations we sorted out a platform of CdLS patients to be further screened by other approaches. Here we report on a refinement of NIPBL mutation scan by MLPA kit. Analysis of 50 pts allowed us to detect three partial deletions of NIPBL gene: two encompassing exon 2 and exon32, and the third affecting exons 1-10. Segregation analysis by DNA polymorphic markers showed that the latter deletion also involves sequences upstream NIPBL. By refined FISH characterization the deletion turned out to extend 2Mb from AGXT2 gene to NIPBL IVS10. It includes 14 genes apart NIPBL, thus featuring a contiguous gene syndrome associated with an extremely severe phenotype of the patient.

A mild phenotype was observed in the pt carrying exon 2 deletion and a severe clinical presentation is present in pt carrying exon 32 deletion.

Application of MLPA to CdLS pts found negative to standard mutation screening is an adjunct tool to disclose full NIPBL mutation spectrum and address genotype-phenotype correlations.

INCREASED THROMBIN GENERATION IN SEVERE HEMOPHILIACS WITH MILD CLINICAL PHENOTYPE

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Introduction. Some severe hemophiliacs (FVIII/FIX<1%) exhibit a mild bleeding tendency, but the basis for this clinical heterogeneity is poorly understood. This study investigated the relationship between the values of Endogenous Thrombin Potential (ETP) and clinical phenotype in severe hemophiliacs. The impact of FVIII/FIX gene mutations and thrombophilic polymorphisms was also evaluated.

Methods. Severe hemophiliacs older than 18 years without inhibitor history and treated on demand were eligible. Mild Bleeders (MB) and Severe Bleeders (SB) were defined as follows: spontaneous bleeding episodes per year ≤2 (MB) or ≤25 (SB) and concentrate consumption <500 (MB) or >2000 (SB) IU/Kg/year. Patients who did not fit these criteria were considered as Intermediate Bleeders (IB). FVIII was measured by chromogenic assay and ETP was measured in platelet-rich plasma after addition of tissue factor.

Results. 22 MB, 22 SB and 28 IB were enrolled. Hemophilia B was more frequent in MB (32%) than in IB and SB (7% and 9%, respectively; $p<0.05$). MB had lower clinical (median 3, range: 0-17) and radiological scores (median 17, range: 3-40) when compared with both IB (median clinical score: 10, range: 0-34; median radiological score: 28, range: 0-48) and SB (median clinical score: 18, range: 10-35; median radiological score: 44, range: 14-62; $p<0.005$). MB showed an older age at first bleed (median 3 yrs, range: 1-10) compared to SB (median 1 yr, range: 0-4; $p<0.005$) and p for trend among the 3 groups was also significant ($p<0.05$). The prevalence of severe FVIII/FIX gene defects (null mutations) was lower (6%) and ETP values were higher (median: 850 nM) in MB compared with both IB (null mutations in 70%; median ETP: 478 nM) and SB (null mutations in 59%; median ETP: 414 nM; $p<0.05$). Non-null mutations and high ETP values were associated with mild phenotype also after adjustment for the type of hemophilia (adjOR: 0.05, 95%CI 0.01-0.45 and adjOR: 0.18, 95%CI 0.04-0.86, respectively).

Conclusions. Our results indicate a low prevalence of null mutations in severe hemophiliacs with mild bleeding diathesis. The measurement of thrombin generation in platelet-rich plasma may allow to identify this subgroup of patients, not otherwise distinguishable by conventional functional assays.

AUTOSOMAL RECESSIVE SPASTIC PARAPLEGIA WITH THINNING OF *CORPUS CALLOSUM* AND PERIVENTRICULAR WHITE MATTER CHNAGES. CLINICAL, MOLECULAR, AND NEUROIMAGING STUDIES

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Hereditary Spastic Paraplegia (HSP) refers to a clinically and genetically heterogeneous Mendelian disorder characterized by weakness, spasticity, and loss of the vibratory sense in the lower limbs. Collectively, HSP accounts for a large set of motor and cognitive handicap in children and young adults. Major progress has been made on the autosomal dominant types of HSP, which account for approximately 70% of cases, but less is known about the autosomal recessive forms of HSP (ARHSP), which appear to be relatively less common and more complex in clinical terms.

We have recently showed that ARHSP-TCC is commonly associated with mutations in SPG11/KIAA1840 on chromosome 15q.

We have now screened a collection of new patients mainly originating from Italy and the Mediterranean basin, in order to further ascertain the spectrum of mutations in SPG11, enlarge the ethnic origin of SPG11 patients, determine the relative frequency at the level of single Countries (*i.e.*, Italy), and establish whether there is one or more common mutation.

In 25 index cases we identified 32 mutations; 22 are novel, including for the first time a large genomic rearrangement. This brings the total number of SPG11 mutated patients in the SPATAX collection to 111 cases in 44 families and in 17 isolated cases, from 16 Countries, all assessed using homogeneous clinical criteria.

While expanding the spectrum of mutations, this larger series also helped to further define clinical and neuroradiological criteria in SPG11-related spastic paraplegia and to corroborate the notion that even within apparently homogeneous population a molecular diagnosis cannot be achieved without full gene sequencing.

TESTING *IN VITRO* AND *IN VIVO* TREATMENTS FOR INCLUSION BODY MYOSITIS

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Evaluation of Nitric Oxide (NO), using the Griess test, in muscle cell cultures obtained from 9 sporadic Inclusion Body Myositis (sIBM) and 6 hereditary IBM (hIBM) GNE-mutated patients, each compared to an age-matched control cell culture, showed that the amount of NO produced by the sIBM myoblasts was always increased compared to the control, but with marked individual variability (20%-80% increase relative to the control cells). In muscle cells from 2 dermatomyositis and 3 polymyositis patients, values between patient and control in a given test were always similar, while in hIBM myoblasts there was a constant 20% increase in NO production. Evaluation of expression of HemeOxigenase-1 (HO-1), a ubiquitous enzyme involved in cell detoxification mechanisms and activated by several factors including NO, showed increased numbers of sIBM cells strongly expressing HO-1 by immunocytochemistry, and, by RT-PCR, increase in HO-1 mRNA levels in sIBM but not in hIBM cells. From June 2006 we have enrolled 13 patients affected by sIBM for simvastatin treatment, and 3 IBM patients are undergoing intravenous immunoglobulin (IVIG 2g/Kg) treatment every two months. Eight patients have reached the daily dose of 40 mg simvastatin and 5 patients are still gradually increasing the dosage. According to the protocol established by the International Myositis Outcome Assessment Collaborative Study (IMACS), the patients are evaluated every two months by manual muscle testing, self-report questionnaires and blood tests for CK, lipids, liver and renal functions. We are also currently validating six core set measures (disease activity measures), developed by IMACS, that capture disease activity and another IMACS core set of "disease damage measures" for assessment of persistent changes in anatomy, physiology, pathology or function. None of the patients have reported clinically significantly side effects during simvastatin treatment. Only one patient has shown a significant CK increase. Three patients have undergone 4 to 6 infusions of IVIG therapy with no relevant side effects, except for 1 patient who developed high blood pressure values during the treatment, controlled with adequate therapy. Regarding clinical evolution, in none of the patients on simvastatin we have observed so far a worsening of the clinical condition, in 3 patients we have observed a slight improvement of muscular strength, while 2 patients refer a subjective improvement of general conditions and in managing common daily activities. No significant clinical variations has been observed in the other patients either on simvastatin or on IVIG every two-months.

IDENTIFICATION OF GENETIC FACTORS RESPONSIBLE FOR RARE DISORDERS WITH CONGENITAL HEART DEFECTS

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Aetiology of Congenital Heart Defects (CHDs) is largely unknown, although mutations in a number of genes have been identified in a few sporadic and familial cases. To investigate the genetic mechanisms underlying CHDs, we collected a cohort of 293 patients, mainly affected by different Conotruncal (CT) CHDs. Sixty patients with CT and 30 with Atrioventricular Canal (AVC) defects were recently enrolled in the study. All patients were screened for NKX2.5, GATA4, FOG2 and GJA5 genes.

Furthermore, two novel cardiogenic genes, TBX20 and GDF1 were analyzed in most of the enrolled patients, while one candidate gene, NFATC1 was screened only in AVC patients. Mutation screening was performed by dHPLC followed by bidirectional sequencing. Screening of GATA4, NKX2.5 and GJA5 genes in the new cohort of TC patient did not identify additional mutations, confirming previous results.

No clear pathogenic mutation was found in the TBX20 and GDF1 genes, although a missense GDF1 change was detected in both patients and controls. NFATC1 gene screening in AVC patients revealed two different missense mutations, which were both absent in 400 control chromosomes.

These results confirm the minor contribute of GATA4, NKX2.5 and FOG2 to Tetralogy of Fallot, and suggest a possible role for GJA5 gene. Screening of GDF1 and TBX20 gene has not supported previous data pointing to a role of these genes in the pathogenesis of CHD. Conversely, our results suggest that mutations in the NFATC1 gene might have a role in AVC pathogenesis.

To evaluate the presence of a locus for non syndromic Absent Pulmonary Valve (APV) on 18q, suggested on personal observations, two subjects affected by isolated non syndromic APV were screened for mutations in NFATC1 gene, which maps to this critical region. No mutation was found by sequencing the entire coding region. Screening of additional genes located in the same genomic region are in progress.

To further evaluate the mutation spectrum associated to Costello Syndrome (CS), Cardio-Facio-Cutaneous syndrome (CFC) and LEOPARD syndrome, causative genes encoding for members of the RAS-MAPK pathway were studied by dHPLC analysis followed by bidirectional sequencing. Mutation screening revealed a causative HRAS mutations in all CS

patients, including a novel in-frame insertion in a four year old subject. According with previous observations, CFC patients were found to be mutated in either BRAF, MEK1 or MEK2 genes.

Preliminary genotype-phenotype correlation analyses indicate that MEK and BRAF gene mutations are associated with overlapping clinical features.

IMPROVING DIAGOSTIC SKILLS FOR INHERITED THROMBOCYTOPENIAS

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The results obtained so far are described according to the specific aims of the project as follows:

Identification and etiologic characterization of inherited thrombocytopenias not yet described. Two new genes, each responsible for novel forms of autosomal dominant thrombocytopenias, have been identified through linkage analysis and mutation screening of the candidate regions (manuscripts in preparation).

Identification of genes responsible for inherited thrombocytopenias that have been described previously but whose etiology is still unknown. We have identified a family with a suspected diagnosis of "gray platelet syndrome". The clinical and morphological platelet features were characterized. A positional cloning strategy is being carried out to identify the gene.

Characterization of the mutations causing inherited thrombocytopenia in Italy. We identified mutations of the c-MPL gene and clonal chromosomal anomalies in five families with Congenital Amegakaryocytic Thrombocytopenia (CAMT). Moreover, we have extended the database of Italian Registry of MYH9-Related Disease (MYH9RD) to 108 patients. In order to validate the presence of MYH9 aggregates in neutrophils as a patognomonic feature of the disease, we are also sequencing the entire MYH9 gene in 30 patients with the clinical features of MYH9RD but with a normal distribution of the protein. We have also excluded the presence of mutations of cytochrome C (CYCS) in 70 patients with features similar to those observed in patients with a detective CYCS.

Identification of genotype/phenotype correlations in patients affected by diseases with known etiology and wide phenotypic variability. In 108 MYH9RD patients we identified a significant correlation between phenotype and genotype at least for the most four common mutations affecting 70% of patients. Thus, the risk of developing kidney failure, cataracts, deafness, and severe bleeding tendency may be predicted. Since some drugs modify the clinical course of kidney damage, patients recognized at risk of renal failure could undergo treatments to prevent or postpone the dysfunction. The genotype and phenotype correlation was also performed in CAMT patients. In this study we did not confirm previous reports and suggested that hematopoietic stem cell transplantation should not be postponed even in those patients whose c-MPL mutations may predict residual activity of c-MPL.

CHARACTERIZATION OF PRELAMIN A FORMS ACCUMULATED IN MANDIBULOACRAL DYSPLASIA AND PROSPECTS FOR THERAPY

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Mandibuloacral dysplasia is a rare autosomal recessive disorder characterized by craniofacial defects, skeletal manifestations including clavicular hypoplasia, acroosteolysis and mandibular bone resorption, cutaneous changes and partial or generalized lipodystrophy.

The mandibuloacral dysplasia form linked to mutations in the LMNA gene encoding lamin A/C has been called MADA, while the form linked to mutations of the prelamin A endoprotease ZMPSTE24 is referred to as MADB.

Both MADA and MADB are characterized by accumulation of prelamin A, which is detected at the nuclear rim and in intranuclear invaginations. Prelamin A is a 74 kDa protein which undergoes subsequent post-translational modifications leading to transient formation of at least four intermediates and ultimately yielding mature lamin A.

The post-translational modifications of prelamin A in MADA had not been so far determined. Here we report the characterization of prelamin A in MADA cells. We show accumulation of different prelamin.

A processing intermediates depending on the passage number, suggesting the onset of a feedback mechanism.

Moreover, we show that treatment of MADA cells with the farnesyltransferase inhibitor FTI-277 or with a combination of mevinolin and trichostatin A is effective in the recovery of the chromatin phenotype altered in MADA, provided that the cells are at low passage number and accumulate carboxymethylated prelamin A.

High passage MADA cells, which accumulated full-length prelamin A, appear to be unaffected by drug treatments.

MOLECULAR CHARACTERIZATION OF A LARGE COHORT OF CORNELIA DE LANGE SYNDROME ITALIAN PATIENTS AND RELATED PHENOTYPES

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Cornelia de Lange Syndrome (CdLS) is a rare multiple congenital disease characterized by facial dysmorphisms, growth/mental retardation, hirsutism, small hands and feet or upper limb reduction defects. Mutations in the NIPBL gene (5p13.2) are responsible of about half of CdLs cases while a small percentage of pts shows molecular defects in the SMC1L1 gene (Xp11.22). SMC3 gene has been associated with CdLS, although its impact on the CdLS syndrome remains to be defined.

We report on the thorough molecular characterization of major genes and submicroscopic genomic rearrangements in a clinically heterogeneous sample of 97 Italian CdLS pts. Mutational screening of NIPBL, carried on by DHPLC and direct sequencing identified 7 missense, 1 in frame deletion, 11 splice-site, 9 nonsense and 13 frameshift mutations, while application of MLPA led to disclose three large multiexon deletions. In selected cases transcript analysis showed the limits of mutation classification at the genomic level, as in two prenatal diagnoses in which the mutation observed in a previous child turned out to be a splicing defect (Q1640Q substitution) or was confirmed to lead to missplicing (c.6108+6T-G, causing the skip of exon 34). Two SMC1L1 mutations were detected, among 36 NIPBL-negative pts and one genomic imbalance and two CNV were disclosed by array-CGH among 24 NIPBL-SMC1L1-negative cases. The stepwise application of this molecular diagnostic flow-chart enabled to disclose the molecular lesion in 51/97 patients (52.5%) also providing a selected cohort of pts to be investigated for candidate genes.

Increasing numbers of molecularly characterised CdLS pts should permit to address genotype-phenotype correlations. Even in the case of NIPBL inactivating mutations, the degree of mental retardation is highly variable, due to different effects of the mutation depending on early or late truncation, and to other unknown genetic and epigenetic factors.

IMMUNOBIOLOGIC AND CLINICAL ACTIVITY OF DNA HYPOMETHYLATING AGENTS IN HUMAN SARCOMAS

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Sarcomas account for approximately 1% of human malignancies and are frequently locally aggressive and/or metastasize. Adjuvant therapy reduces local disease recurrence, although no convincing effects on overall survival have been demonstrated yet; thus, newer treatments are urgently needed for human sarcomas.

In this respect, an appealing option is represented by immunotherapeutic treatments, such as those targeting the Cancer Testis Antigens, that are currently being utilized with promising results in other solid tumors. Moreover, the DNA Hypomethylating Agent (DHA) 5-aza-2'-deoxycytidine (5-AZA-CdR) was recently demonstrated to possess important immunomodulatory properties, among which: i) inducing/up-regulating the expression of CTA in neoplastic cells; ii) functionally reverting the intratumor clonal heterogeneity of CTA expression; iii) up-regulating a set of "immune molecules" (*e.g.*, HLA class I, costimulatory molecules), which positively modulated the recognition of tumor cells by immune effectors.

Based on these notions, we focused at defining the expression of CTA and the immunomodulatory potential of DHA in human sarcomas to eventually design novel therapeutic strategies for this malignancy. RT-PCR analyses on 20 unrelated metastatic sarcoma tissues revealed an overall low frequency of investigated CTA: 10% for NY-ESO-1, 21% for MAGE-A1, 21% for MAGE-A4, 32% for MAGE-A2, 32% for MAGE-A3, 37% for SSX 1-2, 42% for GAGE 1-2, 42% for GAGE 1-6, and 47% for SSX 1-5. The expression of at least one CTA was observed in 74% of lesions, but the expression of CTA appeared clustered: 6 out of 20 metastatic lesions simultaneously expressed over 67% of investigated CTA, while the remaining lesions expressed less than 33% of investigated CTA. To characterize the immunological effects of DHA in human sarcomas, 10 sarcoma cell lines, either established in culture from surgically removed metastatic lesions or available from trade, were treated with scalar doses of 5-AZA-CdR (0.1μM, 0.2μM, 0.5μM, or 1 μM).

Both quantitative RT-PCR and indirect immunofluorescence analyses demonstrated a dose-dependent induction/up-regulation of CTA in sarcoma cell lines, though even the lowest concentrations of the drug were able to generate high levels of CTA expression. Concomitantly, 5-AZA-CdR treatment significantly ($p<0.05$) up-regulated the expression

of HLA class I antigens and up-regulated/induced the expression of ICAM-1 in the panel of sarcoma cell lines under investigation.

These data, though preliminary, strongly suggest that DHA may represent useful therapeutic agents to comprehensively increase immunogenicity and immune recognition of sarcoma cells, providing the rationale for their use in new combined chemo-immunotherapeutic approaches in the sarcoma clinic.

GENETIC ANALYSIS OF ARRHYTHMOGENIC INHERITED DISEASES

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Familial arrhythmogenic diseases such as Brugada Syndrome (BS) are cardiac disorders characterized by electrical ventricular instability that can lead to sudden death at young age. However, to date risk stratification guidelines for Implantable Cardioverter Defibrillator (ICD) implant are not yet comprehensive. Genetic bases have been only partially understood, with four genes covering less than 30% of BS cases. According to the emerging concept of "arrhythmia genomics", the co-segregation of different variants and polymorphisms may modulate the presentation of the clinical phenotype. Our main goal is therefore to develop the molecular analysis of genes associated with arrhythmogenic syndromes both to provide a diagnostic tool for affected patients and their families and to correlate the genotype with the clinical phenotype, in order to improve risk stratification for ICD implant and the management of asymptomatic patients. Our cohort includes now 55 BS patients, carefully characterized from the clinical and electrophysiological point of view. Ten SCN5A gene mutations, including 6 novel variants, have been identified in BS patients (18%). This enabled us to extend the screening to first-degree relatives and diagnose 10 asymptomatic relatives. Since the correlation between genetic variants and clinical outcome requires a longer follow-up, for the moment we were able only to draw few interesting observations, which will have to be confirmed on a larger cohort: the presence of SCN5A mutations seems to be more frequent in symptomatic patients (32% *vs*. 10%, p=0.07); in addition, a spontaneous type I ECG pattern and the inducibility during electrophysiologic study seem to be correlated with the presence of SCN5A mutations. *In vitro* functional studies show a reduction of sodium current for some of the identified SCN5A mutants. In addition, we set up the molecular analysis of other two genes associated with arrhythmogenic syndromes: GPD1L and KCNQ1. The analysis of BS patients to identify possible causing mutations and modifier variants is actually ongoing. Genetic data will be correlated with clinical exams to identify predictors of malignant arrhythmias. The strict interaction between geneticists and clinicians will facilitate the implementation of existing guidelines for ICD implant and the follow-up of asymptomatic patients.

EVALUATION AND REHABILITATION OF SWALLOWING DYSFUNCTION IN PATIENTS WITH RARE NEUROLOGICAL DISORDERS AND MOVEMENT DISORDERS

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In recent years there has been increasing awareness of the feeding difficulties experienced by patients with neurological disorders children with neurodevelopmental disability and adults with movement disorders.

The majority of the studies have been in children with cerebral palsy and in patients with parkinsonism in particular Progressive Supranuclear Palsy (PSP). Many have been found to have major problems with eating and swallowing.

Only a few studies have suggested that nutritional problems are also frequent in patients with inherited neuromuscular disorders. Patients with Duchenne muscular dystrophies, spinal muscular atrophy, congenital muscular dystrophies and other congenital myopathies often have feeding difficulties, gastrointestinal dysfunction and excessive or reduced weight gain but this has not been systematically explored.

Patients with PSP can develop a severe impairment of speech and swallowing. These problems are linked to neuronal damage within the brainstem and their connection to higher brain centres, especially the basal ganglia.

The work will be carried on into 2 workpackages, one focusing on the assessment and rehabilitation of feeding difficulties in the different forms of neuromuscolar disease and the other relating these aspects to patients with PSP and to the various phenotypes observed in the different forms of neuromuscular diseases and top other aspects of clinical management.

The aims of this study are:

- to conduct a survey using a questionnaire on feeding difficulties, gastrointestinal involvement and weight gain in a large cohort of neuromuscular and PSP patients;
- to assess swallowing problems by use of clinical and instrumental tools;
- to correlate these findings with other variables such as age, level of motor impairment, use of ventilatory support and other aspects of clinical management such as scoliosis, heart involvement etc.;
- to evaluate quality of life (qol) and validate in Italian two English language specific questionnaires (swall qol and swall-care questionnaire);
- to suggest therapeutic options and management guidelines according to the results of the research.

Today 10 patients with PSP has been evaluated. Clinical evaluation has been done using the "Progressive Supreanuclear Palsy Rating Scale and Staging System" (Range 0-100; stage 1-5).

Moreover the following instrumentasl evaluations have been carried out: FEES (Fiberoptic Endoscopic Evaluation of Swallowing), Dysphagie Limit, EMG (Elettromiografia).

PCBS AS POSSIBLE EXOGENOUS RISK FACTORS IN THE BLADDER EXTROPHY-EPISPADIAS COMPLEX PATHOGENESIS: THE BLADE PROJECT

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The aim of BLADE is to investigate the possible involvement of Polychlorinated Biphenyls (PCBs) in the pathogenesis of the Bladder Extrophy-Epispadias Complex (BEEC).

BEEC is a multifactorial disorder including congenital malformations of genitourinary tract such as epispadia, bladder extrophy and cloacal extrophy. Environmental contaminants such as Endocrine Disrupting Chemicals (EDC) are suggested as potential risk factors. Among EDC, PCBs deserve special attention because of widespread dietary exposure as well as of mechanisms relevant to BEEC pathogenesis.

PCBs are a numerous and diversified group of more than 200 congeners that can be grouped according to their mechanisms of action, *e.g.*, binding with aryl hydrocarbon receptor. Thus, congener composition is important to understand the potential effects of mixtures of PCB present in human fluids and/or tissues. One study performed within BLADE has characterized the levels and patterns of PCB congeners in the adipose tissue of Italian subjects.

PCB can perturbate cell-to-cell signalling. The external genitalia development is regulated by signal patterns which include members of the Fibroblast growth factor (Fgf) as well as the Bone morphogenetic proteins (Bmp) families; accordingly, we decided to explore the expression of four selected proteins, namely Fgf8, Fgf10, Bmp4, Bmp7, in established cell lines modelling different BEEC targets, following exposure to mixtures of PCB congeners grouped on the basis of possible mechanism additivity.

The first established primary culture cell line has been obtained from smooth muscle *corpora cavernosa* (hFPSM) of 9 and 10 wk fetuses. The establishment of epithelial cells from fetal urethra as well as of primary human bladder smooth muscle and *corpora cavernosa* smooth muscle cells from human adult male is still in progress.

The concentration of each PCB congener to be used in each mixture was derived from the previous results obtained in human adipose tissues. PCBs cytotoxicity was evaluated by the MTS assay in hFPSM following monolayer cell treatments with different concentrations of the mixtures.

None of the concentrations substantially affected cell viability; thus, any effect on cell signalling at realistic exposure conditions is likely unrelated to cytotoxicity.

Western Blot assays are evaluating possible protein modulation of the Fgfs and Bmps target proteins. Preliminary results will be presented.

The work is supported by the project The Bladder Extrophy-Epispadias Complex and Exogenous Risk Factors (BLADE), within the framework of the ISS-NIH Collaborative Programme on Rare diseases (2007-9).

TACKLING RARE DISEASES YET LACKING DIAGNOSIS AND/OR PROGNOSIS: A PILOT PROJECT INTEGRATING DATA COLLECTION AND EXPERIMENTAL STUDIES

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Hepatoblastoma (HB) is the most common primary hepatic tumor in children, with most cases occurring before 2 years of age. Most HB cases are sporadic even though association with other rare diseases (BWS, FAP) have been studied. Subjects with sporadic HBs have a high-frequency of mutations of β-catenin gene. Alteration of IGF2, a peptide hormone crucial for normal development, including enhanced expression and altered promoter-specific transcriptional patterns, have been found in HB as well as in other tumours.

Our integrated study aims: i) to elucidate signalling pathway(s) and molecular mechanism(s) of HB induction and progression with special attention to the role of Wnt/β-catenin and IGF pathways; ii) to characterize possible early markers for HB diagnosis and/or prognosis; iii) to establish a chemically-induced mouse model of HB. Herewith we present the preliminary findings of the project.

Analyses were performed on nine liver tissues samples, obtained from HB patients (aged 8-39 mo) recruited at Bambino Gesù Hospital.

Mutations leading to constitutive activation of β-catenin occur with high frequency in HB and are mainly characterized by point mutations and deletions on exon 3 and its flanking regions. β-catenin gene was amplified and sequenced in all HB liver tissues and no mutations on exon 3 have been detected in tumor tissue. IGF2 gene transcription, is triggered by four distinct promoters in a tissue- and developmental-specific manner, and is controlled by DNA methylation and altered promoter-specific transcriptional patterns frequently occur in cancer. DNA was analysed with Methylation-Specific PCR (MSP) assays and promoter-specific levels of IGF2 transcription has been evaluated by qPCR.

MicroRNAs are short noncoding RNAs that are believed to serve fundamental roles in many biological processes through regulation of gene expression. In order to clarify their role in HB we profiled by an high-throughput method microRNAs expression in HBs and control tissues. We analysed differentially expressed microRNAs and their putative targets

in normal and tumor tissue. Some of these microRNAs, as well as some of their targets, shows an alteration of their expression levels in the analysed samples. Preliminary data show an active role of these microRnas in the Wnt/b-catenin and IGF signalling pathways.

An immunoprecipitation assay for β-catenin was carried out in the tumor tissues and in the normal counterparts from three HB patients. A reduced amount of β-catenin was detected in 2 out of 3 patients. A similar trend was found for c-Jun and Cyclin D1 proteins, both trascriptionally regulated by β-catenin. Interestingly, an increased amount of the growth factor receptor-2 protein (GRB-2), a mitogenic marker, was expressed in HB tumors compared to the normal tissues. Plag-1 protein, whose overexpression may be responsible for IGF-2 expression in HB, was down-regulated in the three HB. Finally, we observed a significant reduction of PDCD4 protein, whose expression is controlled by microRNA-21.

Finally, a chemically-induced mouse model of HB has been established in order to investigate if the molecular events evidenced in the previous WPs in human HB liver tissues, as previously mentioned, occurs also in an animal model exposed to di-2-ethyl-exyl phthalate (DEHP), a plasticizer which, besides being a widespread pollutant, is contained in medical devices used for parenteral nutrition of pre-term neonates: indeed, both pre-term condition and exposure to DEHP have been recently suggested to be environmental risk factors highly associated to HB. Our DEHP-based mouse model shows features to another rodent model of intra-uterine growth retardation (IGF2 knockout in Sprague-Dawley rats), including impaired β-catenin signalling pathways.

This work is performed within the frame of the project "Tackling rare diseases yet lacking diagnosis and/or prognosis: a pilot project integrating data collection and experimental studies" supported by a NIH-ISS 2007-2009 grant.

P. THE ITALIAN NATIONAL REGISTRY OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic Lateral Sclerosis (ALS) is a rare disease included in the Ministerial Decree 279/2001 establishing the National Network for Rare Diseases and the National Registry of Rare Diseases at the Istituto Superiore di Sanità in Italy.

The National Registry of ALS in collaboration with several regional registries (PARALS-Piemonte; SLALOM-Lombardia; SLAP-Puglie) have been activated and are collecting clinical and epidemiological data on ASL.

In this framework, with the contribution of these registries and a specific research project (funded by the Italian Ministry of Health) the National Center for Rare Diseases is implementing the National Registry of ALS (NRALS).

The main aims of NRALS are: i) to collect clinical and epidemiological data on ALS patients at national level; ii) to support basic and clinical research on ALS. The data collected will be the basis to support specific research on ALS focusing on several aspects, including prevalence and/or incidence, risk factors, prognosis and prognostic predictors, pathogenetic mechanisms, and genotype-phenotype correlation.

Has been agreed on data set which includes the informations of the National Register Rare Diseases information and several additional items such as:

- diagnostic criteria El Escorial;
- spinal/bulbar onset;
- inheritance;
- twin status;
- natural history of disease (PEG, tracheostomy, Mechanic ventilation).

The National Registry of ALS is available online (www.iss.it/cnmr).

THERAPY-ORIENTED LARGE SCALE GENOMIC AND GENE EXPRESSION ANALYSIS IN THYMOMAS, MESOTHELIOMAS AND LUNG CARCINOIDS

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Thymomas, mesotheliomas and lung carcinoids are rare tumors and surgical resection is to date the only curative option for their treatment. When surgery is not feasible, common strategies for their cure are missing, since chemotherapy efficacy is often not satisfactory. Due to the low incidence and prevalence of these tumors, drug efficacy and treatment strategy cannot be demonstrated by large scale clinical trials, as suggested by the criteria of "evidence based" medicine.

We proposed a genetic approach in order to identify genes that may play a role in tumorigenesis and, more importantly, that can become effective "drugable" targets, in these different tumor types.

In this context, we performed gene expression microarray analysis in order to identify, within the different tumor types, new categories with specific gene signatures, which are expected to greatly improve the knowledge of the tumors, mainly in terms of disease development and progression (aggressiveness). We started our analysis exploring gene expression profiling among lung carcinoids, since we already had a good collection of samples.

Using a 54,000 probe set (Affymetrix Human GeneChip U133 2.0), we have analyzed a first group of lung carcinoids, constituted of:

- 4 typical carcinoids;
- 4 atypical carcinoids;
- 8 corresponding normal counterparts.

Our preliminary results show that:

- typical carcinoids are relative homogenous according to the detected gene expression profile. On the contrary, atypical carcinoids resulted different from one to another;
- the expression profile of normal lung tissue is homogenous and different from both typical and atypical carcinoids.

These preliminary results are intriguing, so they prompted us to test a higher number of samples. in any case, based on these initial data, we expect to identify a typical-carcinoids-specific signature, evidencing the role of particular pathways that could become new therapeutic targets. atypical carcinoids appear to be a complete different disease and our aim is that to, by enlarging the samples size, identify sub-groups with common features. the experiments are ongoing.

PHENOTYPE CORRECTION OF ADAMTS13 DEFICIENCY AND PROTECTION FROM THE DEVELOPMENT OF THROMBOTIC THROMBOCYTOPENIC PURPURA THROUGH INTRAVASCULAR AND SKELETAL MUSCLE ADAMTS13 GENE DELIVERY IN MICE

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Thrombotic thrombocytopenic purpura, a life-threatening illness characterized by fever, hemolytic anemia, thrombocytopenia, neurological symptoms and renal dysfunction, is caused by deficiency of the Von Willebrand Factor (VWF)-cleaving protease ADAMTS13. Plasma infusion is the treatment of choice for patients with congenital ADAMTS13 deficiency which, however, exposes patients to the risk of infections and volume overload.

Taking advantage of Adamts13$^{-/-}$ mice generated by gene targeting, here we tested the efficacy of a gene therapy approach to restore the level and function of the deficient protein. We constructed an adeno-associated vector containing ADAMTS13 cDNA (AAV-ADAMTS13). AAV-ADAMTS13 efficiently infected human fibrosarcoma HT1080 cells and induced ADAMTS13 mRNA expression as evaluated by RT-PCR. It also induced the expression and secretion of ADAMTS13 protein, as detected by western blot analysis, although at weak level. We tested the recombinant AAV vector *in vivo*. Adamts13-/- mice were administered with 1x1011 vgu of AAV-ADAMTS13 or 1x1011 vgu of AAV-bGal through a lateral tail vein injection.

Two weeks after the treatment, animals were sacrificed and localization of gene expression evaluated by RT-PCR. Systemic injection of the vector resulted in appreciable expression of rADAMTS13 mRNA into the liver, the constitutive site of ADAMTS13 production. By contrast, we could not detect rADAMTS13 mRNA into the kidney and lung of treated mice.

In order to evaluate long term expression of the AAV vector time course experiments were performed. rADAMTS13 mRNA was detected into the liver of treated mice at 4 and 8 weeks from injection and at much lower levels at 16 weeks.

To verify that gene transfer effectively translated in the production of ADAMTS13 protein *in vivo*, antigen levels were measured in plasma of treated mice by ELISA but no protein was detected. Similarly plasma from mice receiving AAV-ADAMTS13 did not show any detectable protease activity, as revealed by a Collagen-Binding-Assay based on

the ADAMTS13 ability to cleave VWF. The lack of detectable levels of the recombinant protein could be due to a low infectivity of the AAV vector.

Indeed, one main limitation of rAAV2 is represented by its native packaging capacity restricted to 4.7 kB, the same size of ADAMTS13 cDNA and this could interfere with the vector infectivity. A recent report described the possibility to obtain more effective vectors using different serotype such as rAAV2/5.

This hypothesis will be tested. We are now going to test the efficacy of adenoviral vectors in producing active ADAMTS13 protein in knockout mice.

P. AMYOTROPHIC LATERAL SCLEROSIS IN ITALY: PREVALENCE AND INCIDENCE BASED ON ADMINISTRATIVE DATA SOURCES

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We combined information from Italian Hospital Discharge Records (HDR) and Death Certificates in order to estimate motor neuron disease occurrence.

32066 HDR with ICD9-CM 335.2_ code were extrapolated from about 60 million in the period 2001-2005. The "hospitalisation paths" of the patients (all the diagnosis and procedures from the first to the last hospitalization) were analysed and a record linkage with Death Certificates was performed in order to exclude unreliable diagnosis and ALS-mimic syndromes. After selection, 9274 patients were considered in analysis.

Incidence was estimated as a mean of new hospitalized cases in 2003-2004 (that is a proxy of incidence in 2002-2003, hypothesizing a 12-months delay from onset to first hospitalization), while prevalence at 01/01/2003 was estimated by summing patients hospitalized for the first time during 2003 (incident cases in 2002) and patients hospitalized before 2003 that were found alive after 31/12/2002 (for a second hospitalization or undetectable among linked Death Certificates).

For Amyotrophic Lateral Sclerosis we found about 1,360 cases hospitalized for the first time during 2003 and 1,390 hospitalized for the first time during 2004, corresponding to a mean incidence rate of 2.4 per 100,000 inhabitants. About 4,000 ALS patients were found alive at 1/1/2003 corresponding to a prevalence rate of 7.0 per 100,000 inhabitants.

Our findings are consistent with those deriving from ALS-Registries.

COMBINED TREATMENT WITH STATINS AND AMINOBISPHOSPHONATES IN MANDIBULOACRAL DYSPLASIA FIBROBLASTS

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Mandibuloacral Dysplasia type A (MADA) is a rare autosomal recessive disorder characterized by craniofacial and skeletal defects, partial lipodystrophy and metabolic alterations. MADA disorder is caused by different homozygous or compound heterozygous mutations falling in the C-terminal of the LMNA gene, encoding lamin A/C. In MADA cells the premature form of lamin A, the prelamin A can not be matured in the functional form and accumulates at the nuclear rim and in intranuclear structures.

This toxic presence is responsible of cellular morphology alterations, cell cycle alteration, chromatin organization and genomic instability. The first post-translational modification of prelamin A is the farnesylation of the cysteine residue to C-terminal domain. The biosynthesis of the isoprenyl groups is part of the mevalonate pathway, so the its disruption by already known inhibitors could be a good strategy to develop *in vitro* and *in vivo* drug therapy.

We decided to test the effects of two different drugs (bisphosphonates and statins) known to act on the same biochemical pathway at different levels. We treated MADA fibroblasts in a two steps model (24 hrs statins treatment and then 12hrs bisphosphonates in a single dose).

This treatment, showed an improvement of the cellular phenotype (reduction of the number of misshapen nuclei and a partial rescue of the heterochromatin organization). Singularly, these drugs were ineffective.

All together these results, suggest that inhibitors of prenylation pathways could be considered as potential drugs.

THERAPEUTIC POTENTIAL OF STEM CELL FACTOR IN THE HUMAN BETA-THALASSEMIA TREATMENT: *IN VITRO* AND *IN VIVO* STUDIES

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In the first part of this project we demonstrated that Stem Cell Factor (SCF) markedly stimulates cell proliferation reactivating fetal hemoglobin (HbF) synthesis in human thalassemic erythroid precursors; pharmacological doses of Dexamethasone (Dex) potentiate these stimulatory effects, thus paving the way for a future clinical application of SCF in beta-thalassemia treatment.

Hence, in order to design a protocol for pre-clinical studies in thalassemic mice, we have performed *in vivo* experiments by evaluating the effects of SCF in C57/BL6 mice treated with high doses of cisplatin, which induces a state of myelosuppression and consequent anemia. This represents an optimal model for evaluating SCF ability to increase haematological parameters such as Red Blood Cell (RBC) number and total hemoglobin content. We observed that, by administering subcutaneous SCF doses of 50 μg/kg 4h before and after cisplatin injection and every 8h for one week, RBCs increased from 7 to 10 $x10^6$/μl and Hb increased from 11 to 14 g/dl in SCF-treated mice, thus reaching normal values. Moreover, preliminary results obtained by HPLC on normal mice treated with SCF±Dex showed that this treatment induces a considerable reactivation of the beta-minor globin chain synthesis. These results will be confirmed on thalassemic mice in the following part of the project.

In parallel, we performed *in vitro* studies in which we analyzed the molecular modifications induced by SCF in normal and thalassemic erythroid precursors grown in unilineage culture of CD34+ hematopoietic progenitors. Our results obtained by RT-PCR showed that in normal erythroid precursors SCF activates a set of genes including Notch2, HES-1, IMPDH2, S100A and HMG (High Mobility Group) family members (HMGI-Y) that play an essential role in the regulation of cell survival and proliferation.

Preliminary investigations performed on erythroblasts derived from two beta-major thalassemic patients showed a stronger SCF mediated induction of Notch 2 as compared with normal control.

The progressive iron overload is the most important complication in beta-thalassemic patients but the relationship among ineffective erythropoiesis and iron-regulatory genes is still unknown. In preliminary experiments we have investigated by Western Blot analysis the protein levels of genes involved in iron metabolism and we found an increase of Transferrin Receptor-1 (TFR-1) and ferroportin levels in SCF-treated cultures of erythroid thalassemic progenitors.

These studies will be further developed in order to clarify the specific role of SCF-induced genes in influencing cell survival, proliferation, HbF reactivation and iron metabolism in beta-thalassemic patients.

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