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**ADVANCES IN MOLECULAR THERAPIES**

**AMT1. In Vitro Evaluation of Type II Ribosome-Inactivating Proteins (RIPs) for Experimental Chemoablation of Muscle Cells in Strabismus and Eye-Movement Disorders**

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**Background:** Today the treatment with botulinum toxin (BTX) is the most used molecular surgery of strabismus and other eye-movement disorders, as alternative to traditional surgery. However the temporary effect of BTX treatment requires the research of alternative therapies. To this purpose the *in vitro* effects of three type II RIPs from plants (lanceolin, stenodactylin and ricin) and of the skeletal muscle-specific immunotoxin saporin-mAb73 were evaluated on muscle cells. **Methods:** The RIPs and the immunotoxin were tested for their cytotoxicity in three cell lines: L6E9 (myoblasts), TE671 and RD/18 (rhabdomyosarcoma), both undifferentiated and differentiated. The aspecific toxicity was evaluated on conjunctival IOBA-NHC cell line. Protein synthesis inhibition, viability and apoptotic changes were assayed. **Results:** All substances showed a strong cytotoxic effect on protein synthesis and viability, with IC50 and LC50 ranging from 0.1 nM to 0.01 µM. Lanceolin and stenodactylin were 1-2 logs more toxic than ricin and 2-3 logs more toxic than the immunotoxin. Myoblasts were particularly susceptible to stenodactylin (IC50<0.01µM). All RIP-treated cells showed typical morphological apoptotic changes and no signs of necrosis. In further experiments miming *in vivo* treatment, no toxic effects were reported on conjunctival IOBA-NHC cell line. **Conclusions:** The strong cytotoxicity observed for stenodactylin at very low dose could be compatible with loco-regional treatments in strabismus and eye-movement disorders. It could be possible to modulate the effect on muscle fibers and to obtain a complete ablation of myoblasts, gaining more durable effects as compared to BTX treatment. Moreover, the absence of necrosis should avoid flogistic side effects.

**AGING**

**AGE1. The Role of Dermal Fibroblasts in the Development of Ectopic Calcifications**

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**Background:** Ectopic calcifications (EC) represent a deleterious consequence of diabetes, renal disorders and aging, being a key determinant of cardiovascular morbidity and mortality. Although the molecular pathways leading to the undesired mineralization of soft connective tissues have been largely investigated in smooth muscle cell cultures (SMC), no effective treatments are available. **Methods:** To further investigate EC, dermal fibroblasts (DF) from healthy individuals and from patients affected by Pseudoxanthoma elasticum, a disease characterized by progressive calcification of elastic fibres, were grown up to 30 days in a standard or in a calcifying medium. **Results:** Degree of mineralization was evaluated after Von Kossa staining, whereas markers of calcification (ALP, ANKH, BMP2, ENPP1, MGP, SPP1) were assessed by RT-PCR and Western Blot. **Conclusions:** Data demonstrate that: 1) DF can be responsible for ectopic calcifications *in vivo*, but, as all other mesenchymal cells, require a specific medium to mineralize *in vitro*; 2) in contrast to SMC, cultured DF do not develop a calcifying signature resembling that of osteoblasts, 3) changes in osteogenic markers are mostly related to the duration of cell cultures, 4) development of a calcified matrix is tightly dependent on the

characteristics of the extracellular environment and the availability of phosphate donor substrates, 5) increased ALP activity is necessary but not sufficient to have mineral deposit formation; 6) the complex balance between pro- and anti-calcifying factors, including circulating factors as fetuin, plays a significant role in the occurrence of ectopic calcifications *in vivo*. Work supported by FCRMO(EctoCal).

**AGE2. Vascular Aging Effect on Medial Aorta Degeneration: Focus on Blood Leukocyte Telomere Length in Hypertensive and Old Patients with Sporadic Thoracic Aortic Aneurysm**

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**Background:** Aging is a well recognised factor in the development of cardiovascular diseases (i.e. sporadic thoracic aortic aneurysm). The physiological aging process determines various changes and a progressive deterioration in structure and function of heart and vascular system, i.e. thoracic aorta. As consequence of these age-related modifications having catalyst and accelerator effect for sporadic thoracic aortic aneurysm (S-TAA), medial degeneration occurs. This pathological entity leads to weakening of aorta wall, which in turn results in aortic dilatation, aneurysm, increased risk for aortic dissections and ruptures. Thus, S-TAA risk increases with chronological as well as biological aging. One optimal marker of this might be peripheral blood leukocyte telomere content. It accurately reflects that of vascular wall and its decrease is associated with premature vascular disease. Thus, the aim of this study was the evaluation of mean blood leukocyte telomere length as predictor for S-TAA. **Methods:** Peripheral blood samples were collected from TAA patients and age- and gender matched controls. Genomic DNA was extracted from leukocytes and telomere length was determined using a chemiluminescence technique. We examined patients and controls selected randomly, but considering the same age and gender. **Results:** A significant lower mean telomere length was detected in TAA group, significantly correlated with age, smoking, hypertension, inflammatory cellular infiltrate and genetic inflammatory variants. **Conclusions:** Thus, telomere assay could contribute to identify individuals at risk for S-TAA. Accordingly, our results should seem to suggest that vascular biological aging might have a strong role in the S-TAA pathogenesis.

**AGE3. Trafficking Profile in Naive and Memory B Cells in Young and Old Subjects**

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**Background:** The impairment of humoral immune response in the elderly human population has been extensively demonstrated. We have reported the increase of a memory B cell (IgG+IgD-CD27-, double negative, DN) population in the elderly, in which there is also a typical inflammatory micro-environment. To evaluate whether this pro-inflammatory status could influence the trafficking phenotype of naive/memory B cells, we have assessed the expression of CCR7, CCR6, CXCR3, CXCR4, CXCR5 and CD62L on naive/memory B subpopulations in young and elderly subjects. **Methods:** We evaluated the expression of some receptors involved in trafficking on different naive/memory B cell subpopulations by flow cytometry approach, using a FACSCALIBUR Cytometer (BD, CA, US). **Results:** In young donors naive/memory B lymphocytes express different chemokine receptors according to the stage of peripheral maturation, whereas the DN B population

express only CXCR3, that leads them to site of inflammation. In the elderly donors this receptor is higher expressed than in young people. Moreover memory switched and DN B cells also express CCR6, which is also involved in the recruitment of cells in the site of inflammation. **Conclusions:** Our data demonstrate that in the elderly naive/memory B cell populations express differently the studied receptors from those observed in young people. This could be discussed in terms of "inflamm-aging." Our hypothesis is that the inflammatory environment, typical of aging, in some way changes the trafficking ability of B cells rendering them more sensitive to the cytokines and chemokines that are over-produced in the elderly.

#### AGE4. Autoantibody Production in Aging: Effect of Cytokine Gene Polymorphisms in Sicilian Ultra-Nonagenarians

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**Background:** Several studies have examined changes in immune functions with advancing age; in particular, the increase in auto-antibodies production might be a marker of the aging associated with a deregulation of immune system. On the other hand, pro- or anti-inflammatory genotypes (particularly cytokine polymorphisms) might impinge upon successful or unsuccessful aging. Here are reported data on the analysis of the effects of cytokine gene polymorphisms on auto-antibody production in aging. **Methods:** We evaluated non-organ specific autoantibodies by an indirect fluorescent antibody test system in a group of ultra-nonagenarians typed for functionally relevant single gene polymorphisms (SNP) of pro- or anti-inflammatory cytokines according to our laboratory procedures. **Results:** Our results demonstrate a significantly increased frequency of anti-nuclear antibody positivity among ultra-nonagenarians bearing the pro-inflammatory 308A TNF allele. Conversely, the percentage of anti-nuclear antibody positivity was significantly reduced among subjects bearing the anti-inflammatory 1082G IL-10 SNP. **Conclusions:** Several studies have largely demonstrated the role of an anti-inflammatory genetic background in the achievement of successful aging. Present results indicate that non organ-specific auto-antibodies production in very old subjects might be a useful marker for the evaluation of the effect of aging associated with reshaping of immune response in subjects bearing a genetically determined pro- or anti-inflammatory profile.

#### AGE5. Age-related Diseases: Key Role of Insulin Resistance for the Association Between Type II Diabetes and Alzheimer's Disease

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**Background:** Alzheimer's disease (AD) and Type 2 diabetes mellitus (T2DM) present many relationships. Insulin resistance (IR) plays a key role in neuronal degeneration and death. Reduced energy makes neurons more sensible to oxidation causing mitochondrial damages. Moreover AD brain has lower insulin utilization, reduced expression of its receptors and of IGF 1 and 2, all necessary for neuronal survival and learning and memory processes. Hyperinsulinemia is correlated with increase of hyperphosphorylated tau-protein. SHIP2, a phosphatase, is an antagonist of PI3K. Since the PI3K plays a key role in the biological effects of insulin, its attenuation could be associated with IR in T2DM. **Methods:** We have conducted a case-control study evaluating the association of three SNPs of SHIP2 in T2DM and AD patients and old and young subjects. SNPs study has been developed by ARMS PCR that make it possible to detect a single SNP thanks to the terminal 3'-nucleotide of one of the primers that anneal with target mutation. **Results:** Significant differences were observed for one functional SNP between AD patients and young subjects, old and young subjects but not AD patients and old subjects.

**Conclusions:** Our preliminary results seem to suggest a putative correlation between this SNP and aging thus strengthening the hypothesis of a close relationship among AD and diabetes. In fact, to verify this relationship we are collecting blood from T2DM patients. Moreover we will collect AD samples because to confirm these results a bigger cohort needs.

#### AGE6. Combination Therapy in Neovascular Age-Related Macular Degeneration

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**Background:** Pathological choroidal neovascularization (CNV) due to age-related macular degeneration (AMD) is a leading cause of legal blindness in people older than 50 years in the Western world. CNV is a multifactorial condition whose pathogenesis involves angiogenesis, inflammation, and fibrosis. All available monotherapies (anti-VEGF agents, steroids, photodynamic therapy with verteporfin (PDT-V)) are directed specifically to only one part of the CNV process. The purpose

of this review is to discuss the current role of combination therapy for the treatment of CNV due to AMD. **Methods:** A MedLine review via PubMed was performed. Evidence available from clinical studies evaluating the use of the combination of anti-VEGFs, steroids and/or PDT-V and from a selective literature search has been considered for this review. **Results:** The results of trials focused on the actual options in the management of neovascular AMD are discussed. Anti-VEGF monotherapy results in a significant increase in visual acuity in patients with wet AMD. The combination of anti-VEGFs with occlusive therapies (PDT-V) potentially offers a reduction of re-treatment rate while maintaining long-term visual benefit. Steroids demonstrated an antiangiogenic effect, targeted the extravascular components of CNV such as inflammatory cells and fibrocytes and seems to be efficacious in patients non-responder to anti-VEGF monotherapy. **Conclusions:** Combination therapy has been proposed to interfere with the multiple stimuli to pathologic vascular proliferation. Many experiences have been conducted and showed encouraging results. Although there is a strong rationale for applying multiple combined therapy in the treatment of CNV, further study is required to determine correct combinations and dosage.

#### AGE7. Mediterranean Diet and Longevity

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**Background:** The effect of calorie restriction on human health has been debated due to the lack of information and appropriate study. Furthermore, in many population-based studies and randomized trials there are evidences that a dietary pattern rich in some nutritional food groups such as fruits and vegetables plays a role in delaying age-related diseases. In the inner part of Western Sicily we have some "blue zones" where the ratio of centenarians vs. total population is higher (4.32) than in the Italian population (2.4). Those "blue zones" are located far from the sea in the area of Sicani Mountains. **Methods:** The people that we interviewed are female and male centenarians belonging to several villages that underwent many analyses: hematological, chemical analysis, complete anamnesis, ADL, MMSE and MNA nutritional assessment tests. Furthermore oxidative stress assessment, such as ROS and NOS, were performed. Also dietary intake, through 24 hours recall has been recorded and different levels of adherence to the Mediterranean diet observed. **Results:** The results taken together showed a good control of hematological and chemical parameters of healthy status and good adherence to Mediterranean diet, which seems to play a key role in diseases prevention. **Conclusions:** Mediterranean diet might play a key role in disease prevention and for management of age-related diseases. To reach successful aging it is advisable to follow a diet with low quantity of saturated fat and high amount of fruits and vegetables rich in phytochemicals.

#### AGE8. Impact of Smoking, Alcohol Consumption and Aging on Antioxidant/Pro-Oxidant Balance in Age-Related Macular Degeneration

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**Background:** Oxidative stress, inflammation, and genetics are thought to contribute to the development of age-related macular degeneration (ARMD), the most common cause of blindness in the elderly. The aim of this study was to determine whether smoking, alcohol consumption and aging, which constitute the main exogenous sources of reactive oxygen species (ROS), affect the balance between oxidant production and antioxidant levels in ARMD. **Methods:** Superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT) activities as well as malondialdehyde (MDA), protein carbonyl (PC), 8-hydroxy-29-deoxyguanosine (8-OhdG) and total oxidation status (TOS) levels, were measured in patients with early ARMD (n=211) and late ARMD (n=205), and control persons (n=262). **Results:** When compared with healthy controls, early- and late- ARMD patients showed significant decreases in the activities of SOD and GSHPx, but not CAT, along with marked enhancements of MDA, PC, TOS and 8-OhdG ( $P < 0.01$ ). No notable differences were observed in the early- versus the late-ARMD group for each of the above-mentioned dependent variables. Multiple regression analysis revealed that in healthy subjects chronic smoking and aging had the strongest impact on oxidative stress parameters, whereas in ARMD patients, the combination of smoking, drinking, and aging was the greatest predictor of oxidative DNA, protein and lipid damage. **Conclusions:** Cigarette smoking, alcohol consumption and aging could be aggravating factors contributing to serious redox imbalance and oxidative damage in ARMD. Identification of factors exacerbating ARMD-associated oxidative stress can facilitate development and adoption of effective preventative measures for this disease.

**AGE9. BPIFB4 Missense Variants Associate with Exceptional Longevity in Independent Populations and Influence Cell Signaling**

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**Background:** Centenarians, despite being exposed to the same environmental conditions as members of the average population, manage to live much longer. A recent Genome-Wide Association Study (GWAS) for exceptional longevity in Southern Italian Centenarians (SICs) identified four SNPs that were either non-synonymous or non-synonymous taggers with a  $P < 1 \times 10^{-4}$ . **Methods:** A two-stage genetic association study on long-living individuals and controls, followed by *in vitro* and *ex vivo* kinase activity detection of mutated and wild type BPIFB4. **Results:** We identified rs2070325 (BPIFB4-Ile268Val) consistently associated with human longevity under recessive model in Italian, German and US cohorts. Rs2070325 is in strong LD ( $r^2=0.93/D'=0.98$ ) with rs2889732 (BPIFB4-Asn320Thr) and *in vitro* studies show that BPIFB4 is detected in cytoplasmic vesicles and secreted together with 14-3-3. HEK293T transfections with Ile268Val/Asn320Thr BPIFB4 activate PKC alpha, AMPK, p65/RELA and improves BPIFB4/14-3-3 cellular secretion, counteracting PKC alpha, AMPK and p65/RELA inhibition observed after wild type BPIFB4 transfection. **Conclusions:** Further studies are needed to understand why these mutations are protective for human health.

**AGE10. In Situ Determination of Epigenetic Mechanisms at Work in Soft Elastic Tissues during Aging**

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**Background:** Epigenetic modifications, such as DNA methylation and histone modification, are largely responsible for the variable activation and repression of specific genes at specific time points during cell lifespan. Thus, epigenetic mechanisms have been described as crucial processes for embryonic development and deregulation leading to malignant transformation or senescence and aging. More recently, epigenetic imprinting has been associated to stem cell reprogramming and therefore to tissue growth and regeneration. **Methods:** The methylation of mammalian genomic DNA mainly occurs in GC rich regions termed CpG islands. Methylation of cytosine residues is catalyzed by DNA methyltransferases (DNMTs), which encompass three active isoforms: DNMT1, DNMT3a and DNMT3b. DNMT1 is mainly associated to the maintenance of the methylation pattern on the daughter strand during DNA replication, whereas DNMT3a and DNMT3b are powerful de novo methyltransferases. **Results:** In our studies, we have focused on the epigenetic imprinting that could affect the differentiation of elastic tissues, as a hallmark of well formed and healthy tissues, a status that must be reached to heal, to reconstruct and to regenerate soft tissues. Though epigenetic mechanisms have been poorly investigated for such tissues, GpC rich motifs allowing Sp-1/Sp-3 binding on extra cellular matrix genes have been documented. **Conclusions:** Our recent experiments confirmed the importance of such GC rich regions on the methylation status of promoters driving genes encoding elastic fiber-related elements. Several technical approaches have been proposed to be as close as possible of the native tissue, including the determination of CpG methylation pattern from embedded human tissues.

**AGE11. Involvement of Oxysterols in the Pathogenesis of Alzheimer's Disease**

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**Background:** Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the extracellular accumulation of amyloid-beta (A $\beta$ ) in neuritic plaques. Altered cholesterol metabolism in the brain has been suggested to be implicated in pathogenesis of AD. **Methods:** We have investigated the potential interaction between the oxysterols 24-hydroxycholesterol (24-OH), 27-hydroxycholesterol (27-OH) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), present in the brain, and A $\beta$  in human differentiated neuronal cell lines, SK-N-BE and NT-2. Expression and synthesis of CD36 and  $\beta$ 1-integrin receptors were analyzed by real time RT-PCR and Western blotting. The necrotic, apoptotic and redox parameters were measured by microscopic and biochemical analysis. **Results:** Our studies show that all three oxysterols enhance the binding of A $\beta$  to neuronal cells by up-regulating expression and synthesis of CD36 and  $\beta$ 1-integrin receptors, which both form, with CD47 receptor, the multireceptor complex that mediates the A $\beta$  binding to the plasma membrane of neurons. However, only 24-OH markedly potentiates the proapoptotic and proneurogenic effects of A $\beta$  on cells, likely through a strong

enhancement of reactive oxygen species' (ROS) generation and impairment of cell redox equilibrium. Cell incubation with antioxidants quercetin or genistein prevents 24-OH's pro-oxidant effect and potentiation of A $\beta$ -induced necrosis and apoptosis. Thus, the presence of 24-OH in the close vicinity of amyloid plaques appears to enhance the adhesion of large amounts of A $\beta$  to the plasma membrane of neurons, then to amplify the neurotoxic action of A $\beta$  by locally increasing ROS steady-state levels. **Conclusions:** These results support a primary involvement of altered brain cholesterol metabolism in the complex pathogenesis of AD.

**AGE12. Interaction Between 4-Hydroxynonenal and Amyloid- $\beta$  in Amplifying Neuronal Damage**

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**Background:** Alzheimer's disease (AD) is characterized by intracellular neurofibrillary tangles made of hyperphosphorylated tau and senile plaques made of extracellular deposits of amyloid  $\beta$  (A $\beta$ ). Lipid peroxidation is considered as primarily involved in the pathogenesis of AD. One of its more reactive end-products, 4-hydroxynonenal (HNE), has been proposed as a candidate biomarker of AD because it was found in the brain of AD patients, in both neurofibrillary tangles and in senile plaques, as well as in the cerebrospinal fluid. HNE production in the brain is stimulated by A $\beta$  and, conversely, A $\beta$  formation is up-regulated by HNE. **Methods:** We applied a precursor-cell-based approach, comprising primary cultures of human dental-pulp progenitor cells, which spontaneously differentiate to neuron-like cells *in vitro*. Membrane receptor gene expression was quantified by real time RT-PCR. Amyloid  $\beta$  internalization was observed by Congo red staining. Necrosis was evaluated by measuring the extracellular percentage of lactate dehydrogenase. **Results:** Our findings point to the ability of HNE and A $\beta$  to interact, with consequent potentiation of A $\beta$ 's cytotoxicity. HNE was found to cause overexpression not only of CD36 but also of another component of the multireceptor complex that binds the A $\beta$  peptide, namely  $\beta$ 1-integrin. The up-regulation of these components allowed the neurotoxic peptide to accumulate in greater amounts within the cells, and to induce much more extensive cell death than occurred in cells challenged with A $\beta$  alone. Cell death was completely prevented by the specific receptor blockade. **Conclusions:** These results support the involvement of HNE in the pathogenesis of AD.

**BIOMARKERS FOR (NON-CANCER) DISEASE DETECTION**

**BM1. HepG2 Spheroids as *in Vitro* Model to Study the Release of Gamma-glutamyltransferase Fractions**

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**Background:** Four gamma-glutamyltransferase (GGT) fractions (b-, m-, s-, and f-GGT) have been described in plasma. Clinical study on healthy subjects and patients affected by non-alcoholic or alcoholic liver disease or chronic viral hepatitis showed that s-GGT fraction is a marker of hepatocellular damage, whereas b-GGT is an index of liver metabolic dysfunction. **Methods:** Aim of this study is to deepen the mechanism of fractional GGT release by hepatocytes. For this purpose we used HepG2 cells cultured as spheroids. Morphology, biochemical parameters (GGT activity, protein content), cellular GGT distribution (immunohistochemistry) were evaluated over a period of 15 days. Secreted GGT fractions were quantified by gel-filtration chromatography associated with a GGT-specific post-column reaction. **Results:** HepG2 spheroid formation can be divided in two stages, immature (1-6 days) and mature (>6 days). In the media of immature spheroids only b-GGT [mean (SD), 1.31 (0.01) U/L] and f-GGT [0.45 (0.01) U/L] were present. Between day 6 and 9, f-GGT [3.49 (0.36) U/L,  $P < 0.001$  vs. day 6] and s-GGT [0.25 (0.02) U/L,  $P < 0.001$  vs. day 6] increased significantly, whereas b-GGT activity was unchanged [1.04 (0.08) U/L]. Morphological and immunohistochemical analysis showed the presence, in mature spheroids, of structure compatible with bile-canaliculi surrounded by GGT protein. s-GGT fraction was non-detectable in media obtained from HepG2 monolayer culture. **Conclusions:** In conclusion, mature HepG2-spheroid culture is a good model for the *in vitro* study of fractional GGT release. Obtained results showed that the 3D structure is necessary for the secretion of s-GGT, which could result from an extracellular modification inside bile-canaliculi.

**BM2. YKL-40 (Versus COMP) as a New Marker of Knee Osteoarthritis**

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**Background:** The identification and study of markers of osteoarthritis is an established goal of the scientific community. The purpose of this work is to point out the correlations between YKL-40 and COMP, two glycoproteins involved for a long time, as markers in the study of the osteo-cartilagineous degenerative process. The efficacy of YKL-40 as a serum marker of cartilage has been demonstrated in patients with knee osteoarthritis (OA), correlating the variations of the same group with viscosupplementant treatment and with COMP serum. **Methods:** 35 patients, divided into two groups, were recruited for the study. The first group was administered a cycle of 5 intra-articular injections of hyaluronic acid while the second group received a cycle of 5 injections of polynucleotide gel. Patients were evaluated by means of end-points including the KOOS scale and VAS. **Results:** In all the three groups there is indiscriminately a decrease in pain symptoms. In the second subgroup of patients (moderate osteoarthritis) the amount of YKL-40 at 3 weeks decreased significantly ( $P = 0.0473$  CI 95%). After 5 weeks, in the same subgroup, a significant decrease of COMP ( $P = 0.0003$  CI 95%) was noted. As for COMP, YKL-40 seems to be a valid biomarker in evaluating the severity of the initial osteoarthritis; it also appears to be effective in monitoring the clinical course of patients with moderate osteoarthritis. **Conclusions:** The two proteins appear to describe different aspects of osteoarthritis: YKL-40 is more sensitive in monitoring short-term variations, whereas COMP is more suitable to monitor the long term.

**BM3. Simultaneous Detection of Circulating Biomarkers of Oxidative Stress and Endothelial Dysfunction in Patients with Psoriatic Arthritis (PsA)**

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**Background:** Psoriatic arthritis (PsA) is a chronic inflammatory arthritis associated with significant disability and increased cardiovascular mortality. Enhanced oxidative stress and endothelial dysfunction may contribute to the increased cardiovascular risk in patients with PsA. To ascertain simultaneous presence of oxidative stress and endothelial dysfunction in PsA patients, we measured plasma levels of malondialdehyde (MDA, marker of lipid peroxidation), protein carbonyls (ProCa, marker of protein oxidation), asymmetric dimethylarginine (ADMA, marker of endothelial dysfunction), and L-arginine and evaluated L-arginine/ADMA ratio as a measurement of endothelial dysfunction. **Methods:** Twenty patients with PsA without any signs of cardiovascular disease and 20 healthy controls were recruited for our study. PsA patients were matched for age, sex and disease duration. Fasting venous blood samples were obtained and plasma was used for determination of MDA with high-performance liquid chromatography (HPLC), ProCa with fluorescein thiosemicarbazide fluorimetric assay, ADMA and L-arginine with HPLC. **Results:** Plasma MDA and ProCa levels were significantly higher in PsA patients than in healthy controls ( $1.43 \pm 0.09$  micromol/L and  $0.24 \pm 0.02$  nmol/mg proteins vs. respectively  $0.57 \pm 0.08$  micromol/L and  $0.10 \pm 0.02$  nmol/mg proteins,  $P < 0.001$ ). **Conclusions:** In conclusion, increased oxidative stress coupled with endothelial dysfunction (as shown by increased plasma ADMA levels and reduced L-arginine/ADMA ratio) is present in PsA patients.

**BM4. Dopaminergic Receptor Gene Polymorphisms in Children Affected by ADHD/ASD Overlapping**

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**Background:** Several studies have shown a genetic role in the pathogenesis of many childhood psychiatric disorders. The most common childhood psychiatric disorder is the attention deficit hyperactivity disorder (ADHD) and some reports showed the co-occurrence in ADHD children and Autism Spectrum Disorders (ASD). Many investigators focused their attention on polymorphisms affecting gene regions coding for dopamine receptors. In this study we evaluate the association of three single-nucleotide polymorphisms (SNPs) with clinically significant level of autistic symptoms among children with ADHD. **Methods:** We enrolled 150 children who were divided into four groups: children with ADHD, children with ASD, children with co-occurrence of ADHD/ASD, and control subjects. We investigated rs265975 C/T (174862195C>T) for dopamine receptor D1 gene, rs1076560 C/A (113283688C>A) and rs1079597 G/A (113296286C>T) for dopamine receptor D2 gene utilizing previous DNA extraction and amplification, restriction enzymes that recognized one of two allelic variants. **Results:** Our results demonstrated that homozygosity T/T for rs265975 had a lower frequency in ADHD patients compared to other groups,

whereas small differences were seen in heterozygosity C/T. Both heterozygosity C/A for rs1076560 and heterozygosity G/A for rs1079597 showed higher frequency in ASD group with respect to control children and ADHD patients, whereas in ADHD/ASD group a ratio 3:1 vs unaffected people was seen. The same trend, but with slight differences, was observed in homozygosity A/A for rs1076560 and rs1079597. **Conclusions:** These preliminary data pointing to differences between ADHD/ASD and other groups must be confirmed and encourage us to enlarge our study populations.

**BM5. Performance of CD64 Index and Soluble TREM-1 as Biomarkers in Late Onset Sepsis of Preterm Neonates**

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**Background:** In Neonatal Intensive Care Units, infections remain important cause of neonatal morbidity and mortality. Early and accurate diagnosis improves the outcome. The aim of this study was to explore the performance of CD64 index and soluble TREM-1 as biomarkers of sepsis in a group of preterm neonates. **Methods:** We enrolled 54 neonates with gestational age < 32 weeks and weight < 1500 gr. Sixteen newborns (septic group) developed late onset sepsis (culture positive) during the 16th to 25th days of life (T1); they were also tested between the 5th and 15th days of life (T0, free from infection). Thirty-eight preterm, without clinical and laboratory signs of sepsis, were tested between day 5 to 20 of life as laboratory normal range. Fifty-microliter blood samples were processed using the Leuko64 Assay kit and analyzed with an Abbott Cell-Dyn Sapphire hematology analyzer. Soluble TREM-1 was quantified using sandwich immunoassay kit. Statistical analysis was performed by Medcalc. **Results:** Clinical and laboratory data, whole blood leukocytes, neutrophils, monocytes and platelet counts were comparable in all the groups/conditions, besides a significant increase ( $P = 0.049$ ) of neutrophils in septic group (T0 vs T1). CD64 index increased significantly in septic group T1 vs T0 ( $P = 0.0002$ ) and T1 vs control group ( $P = 0.0001$ ) and ROC curve indicated cut-off 2.86, sensitivity 87.52%, specificity 97.1%, AUC 0.925. Soluble TREM-1 didn't show any differences in septic group (T1 vs T0  $P = 0.9$ ) and T1 vs control group ( $P = 0.8$ ). **Conclusions:** In preterm neonates CD64 index can be useful as biomarker of late-onset sepsis whereas soluble TREM-1 showed less efficient diagnostic role.

**BM6. Senescence Markers**

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**Background:** Evolutionary theory and empirical evidence suggest that aging is a process of gradual accumulation of damage in cells and tissues of the body. The progressive loss of ability to interact effectively with environmental stimuli is accompanied by progressive modification and adaptations that are influenced by lifestyle and genetic background of the individual. These affect the ability to age successfully, defined both as longevity and/or escaping the major age-related diseases. Some of the most important characteristics of adaptive immunity in aging are compatible with this assumption. Actually, the antigenic load results in the progressive generation of a chronic low grade inflammatory response involved in body and brain aging. **Methods:** Data on genetic background and immune system have been obtained in the last 10 years studying Sicilian centenarians and subjects affected by aging related diseases. **Results:** Studies have been focused on the genetic background predisposing to aging related diseases. Data gathered on gene variants in cytokine, pathogen-related pattern receptors or acute phase response genes allow the research group to define a complex trait in which the antagonistic pleiotropy of regulating immune-inflammatory mechanisms might play a central role in predisposing to a large array of age-related diseases and in determining lifespan expectancy. **Conclusions:** These findings suggest that different alleles at different immune-related genes coding for pro- or anti-inflammatory molecules may affect individual life-span expectancy and might be useful markers for the evaluation of aging-associated disease risk.

**BM7. Training Effects on Laboratory Parameters Are Independent of Genetic Polymorphisms of IL-10 and TNF-alpha (TNF-α)**

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**Background:** It is well known that exercise has beneficial effects on health. Although intense exercise is experienced by the body as a condition of stress, a well designed training has long term beneficial effects on the organism of an athlete. Less is known about the effects that the genetic background might have on training

adaptation and on the consequent modification of laboratory parameters. **Methods:** In our study we evaluated the blood chemistry parameters of a group of 41 athletes compared with a group of 45 amateur athletes, to assess whether the training has effects on their variation. In addition we typed our subjects for polymorphisms 308 A/G of the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and 1082 A/G of Interleukin-10 (IL10). **Results:** After statistical analysis, performed with Mann-Whitney Test, we observed a statistically significant ( $p$  value < 0,05) increase of basophils, eosinophils, monocytes, and total bilirubin and decreased levels of neutrophils, glucose, electrolytes and AST in professionals compared to amateurs. These parameters were not modified by the genetic background. Actually the training modification observed were independent of the presence of pro-inflammatory (carrier allele A of 1082 A/G of IL10) or anti-inflammatory alleles (subjects A negative for 308 A/G of TNF $\alpha$ ). **Conclusions:** The genetic polymorphisms analyzed do not influence changes in laboratory parameters values induced by professional training.

**BM8. TGF- $\beta$  Pathway Polymorphisms as Markers for Gender Differential Susceptibility to Sporadic Thoracic Aortic Aneurysm**

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**Background:** It has become increasingly evident that the immune system plays a pivotal role in the development of thoracic aortic aneurysm (TAA). The Transforming Growth Factor (TGF) - $\beta$  isoforms might be involved in TAA pathogenesis inducing disruption of extracellular matrix, apoptosis of smooth cells in tunica media, metalloprotease production and remodelling tissues after inflammation. **Methods:** 133 subjects affected by TAA (from Cardiac surgery Unit of Palermo University Hospital), 107 unrelated patients of the same unit without TAA and a group of 91 healthy controls matched for age and gender, all living in western Sicily, were typed for TGF- $\beta$ 1, TGF- $\beta$ 2 and their receptors polymorphisms by a KASPar assay (the KBiosciences Competitive Allele-Specific PCR SNP genotyping system). Genotype and allele frequencies were compared by statistical analysis. **Results:** No differences in distribution between cases and controls were observed except for TGF- $\beta$ 2 rs900 TT genotype, whose frequency was increased in patients affected by aortic aneurysm in comparison to the controls ( $P = 0.037$ ). In particular this genotype was significantly increased in women affected by TAA in comparison both to women of control patient group ( $P$  value = 0.042) and of health control group ( $P = 0.010$ ). **Conclusions:** TAA is a complex pathology with a greater prevalence among men. Our results suggest that rs900 TGF- $\beta$  SNP might be a genetic factor involved in women's susceptibility to TAA.

**BM9. Evaluation of Genome-Wide Expression Profiles of Blood Neutrophils in Cystic Fibrosis Patients Before and After Antibiotic Therapy**

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**Background:** Cystic fibrosis (CF) lung disease is characterized by massive extravasation of neutrophils into the airways that undergo apoptosis and thereby do not clear respiratory infections. The surrogate end-points that describe this process and the effect of antibiotic therapy, such as spirometry, are not sensitive and non-specific. We sought to evaluate the gene expression profile of circulating neutrophils in CF patients before and after antibiotic treatment. **Methods:** Microarray analysis (28,869 genes, Affymetrix GeneChip Gene 1.0 ST Array System) was performed in blood neutrophils from 10 CF patients before and after treatment for clinical exacerbation with antibiotics and 7 healthy control subjects. **Results:** Blood neutrophils before therapy presented 293 down-regulated genes and 57 up-regulated genes as compared with control subjects (considering as cut-off  $P < 0.05$  by ANOVA). Comparison between the same patients before and after therapy (with the same cut-off by paired t test) showed instead that 1,422 genes were down-regulated and 282 up-regulated following antibiotic treatment. Interestingly, three genes appeared to be sensitive to therapy and returned to "healthy" condition: phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*), hydrogen voltage-gated channel 1 (*HVCN1*), and dom-3 homolog Z (*DOM3Z*). The up-regulation of these genes after therapy were confirmed by RT-PCR in blood neutrophils ( $n=5$ ) and in sputum neutrophils obtained from the same patients ( $n=7$ ). **Conclusions:** These results suggest the feasibility of investigating novel biomarkers of therapeutic efficacy by a global gene-wide platform and indicate more specific targets for future interventions involving respiratory burst and apoptosis.

**BM10. First Trimester Biochemistry to Predict Cesarean Section for Fetal Distress and Cardiocotographic Alterations During Labor at Term of Gestation**

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**Background:** At this time the literature tries to predict the outcome of pregnancy in the first trimester of pregnancy such as analyzing placental function. Our study compares first trimester clinical and biochemical characteristics between controls and pregnancies affected by cardiocotographic (CTG) anomalies or characterized by urgent Cesarean section (CS) during labor at term. **Methods:** In this study at term pregnancies were considered. Clinical and biochemical characteristics were evaluated during the first trimester. The blood examinations have been performed between 2004 and 2010. We collected data about all CTGs performed during labor classified in three categories based on the National Institute of Child Health and Human Development terminology of 2008: 1)normal; 2)intermediate; 3)abnormal. **Results:** In a multivariate logistic regression reduced PAPP-A is correlated with a higher frequency of urgent CS at term of pregnancy ( $P < 0.05$ ), regardless of hypertensive disease of pregnancy, IUGR, BMI, maternal age, gestational age at delivery and other obstetric pathologies considered. Abnormal CTGs were associated with older maternal age, higher prevalence of nulliparous women, and lower placenta weight than normal ones ( $P < 0.05$ ). First minute Apgar scores were lower in category 3 than in 1 and 2 ( $P < 0.05$ ). Finally, CTG alterations defined in category 3 were correlated with lower free-beta-hCG and PAPP-A values during the first trimester, although without statistical significance. **Conclusions:** A low PAPP-A in the first trimester of pregnancy appears to be correlated with a higher frequency of urgent CS at term of pregnancy and fetal distress during labour.

**BONE METABOLISM**

**BMT1. Nitric Oxide Mediates Low Magnesium Inhibition of Osteoblast-Like Cell Proliferation**

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**Background:** An adequate intake of magnesium is important for bone cell activity and contributes to the prevention of osteoporosis. Because i) magnesium is mitogenic for osteoblasts and ii) reduction of osteoblast proliferation is detected in osteoporosis, we investigated the influence of different concentrations of extracellular magnesium on osteoblast-like SaOS-2 cell behavior. **Methods:** SaOS-2 cells were cultured in media containing different concentrations of magnesium. Nitric oxide synthase (NOS) activity was evaluated by mass spectrometry and Griess assay. NOS isoforms were studied by western blot. **Results:** We found that low extracellular magnesium inhibited SaOS-2 cell proliferation. An additive effect was observed when cells cultured in low magnesium were silenced for the magnesium transporter Transient Receptor Potential Melastatin (TRPM)7, which plays a prominent role in intracellular Mg homeostasis. In particular, we found that low magnesium inhibition of SaOS-2 cell proliferation was due to an increase of nitric oxide production through the up-regulation of inducible nitric oxide synthase (iNOS). Indeed, both pharmacological inhibition with the iNOS inhibitor L-NIL and genetic silencing of iNOS by siRNA restored the normal proliferation rate of the cells. **Conclusions:** Because a moderate induction of nitric oxide is sufficient to potentiate bone resorption and a relative deficiency in osteoblast proliferation can result in their inadequate activity, we conclude that maintaining Mg homeostasis is relevant to ensure osteoblast function and, therefore, to prevent osteoporosis.

**BMT2. Fragility Fractures and High Energy Fractures: Serum Concentrations of IL-6, TNF- $\alpha$ , OPG, RANKL and Their Correlation with Radiographic Assessment**

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**Background:** Stages of bone turnover during fracture repair can be assessed employing serum markers of osteoblastic and osteoclastic activity, inflammatory cytokines, clinical evaluation and imaging instruments. Our study compares the fracture healing process in fragility fractures and high energy fractures by evaluating serum changes of IL-6, TNF- $\alpha$ , OPG and RANK-L in combination with radiographic (RUST) and clinical (LEM) assessments. **Methods:** Subjects: femoral or tibial shaft fractures (group A,14), femoral fractures (group B,14), healthy (control A,14) and osteoporotic subjects (control B,14). Serum concentrations of IL-6, TNF- $\alpha$  were

quantified by Quantikine R&D Systems, RANK-L by BioVendor and OPG by Biomedica Medizinprodukte. **Results:** Results showed a significant decrease in IL-6 and TNF- $\alpha$  during fracture healing, with their values higher in group A than B and lower in both controls compared to T0 (before surgery). OPG was significantly lower in each control group than that of the respective fractured group. In addition, OPG at T0A was significantly lower than at T0B whereas at T10A (after 10 weeks) OPG was less than at T10B. RANKL was significantly higher at T10 than at T0 only in group B. RANKL/OPG ratio was significantly higher in both controls than in fractured groups and significantly increased at T10. IL-6 and TNF- $\alpha$  correlated with RUST and LEM in fragility fractures and high energy fractures, whereas RANKL/OPG ratio was associated with these parameters only in fragility fractures. **Conclusions:** Our findings suggest that these serum parameters might be used to assess the stages of fracture healing. Further studies are required to clarify the complex fracture healing process.

**BMT3. FokI Polymorphism of the VDR Gene and Its Association with Intervertebral Disc Degeneration-Related Pathologies in the Italian Population**  
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**Background:** Lumbar disc degeneration (LDD) is the main cause of low back pain and has a complex etiology. Several risk factors likely contribute to the genesis and/or acceleration of disc degeneration, however, there is some evidence for an influence of genetic factors and familial predisposition. The vitamin D receptor (VDR) gene is one of the most studied genes involved in the predisposition to LDD. However, the data on the interplay between VDR genotypes, environmental factors and specific spine pathologies are still controversial. **Methods:** By using a case-control design, 234 Italian LDD patients and 70 healthy controls were enrolled. MRI-based patients' clinical assessment was performed and a questionnaire assessing constitutional and environmental risk factors was obtained. Blood samples were collected, genomic DNA was extracted and VDR *FokI* polymorphism was detected by PCR-RFLP. **Results:** Preliminary data showed that the genotypes frequencies for *FokI* polymorphism, in patients versus controls, were 44.9% versus 35.7% for FF homozygosity, 44.9 versus 51.4% for Ff heterozygosity, 10.3% versus 12.9% for ff homozygosity, respectively. A significant association was found between the FF genotype and disc herniation (OR=1.82; CI=1.02-3.27), with 50.3% of patient with hernia versus 35.7% of controls presenting the FF genotype. **Conclusions:** The *FokI* FF genotype is a putative risk factor for disc herniation, but a larger control group is needed to better define the genotypes frequencies in the normal population. Moreover, study of other VDR polymorphisms (*TaqI*, *BsmI* and *ApaI*) is in progress together with the evaluation of the vitamin D status and the association with constitutional and environmental factors.

**CARDIOVASCULAR BIOMARKERS**

**CVBM1. Circulating Lipid and Lipoprotein Levels Distinguish a Gender-Related Response to Statins in Subjects in Primary and Secondary Prevention**  
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**Background:** Cardiovascular risk in men rises around the fourth decade of life, whereas women appear to be protected by sex hormones until menopause, which however negatively affects the lipid profile and the related cardiovascular risk. Since the 1980s, the incidence of cardiovascular disease has been reported to progressively decline in men, but it persists almost unchanged in women. Major clinical trials (e.g., on statin therapy) have mostly been conducted in men and have fostered the introduction of these agents into clinical practice worldwide. Only a few reports have however evaluated a possible differential activity of statins in the two genders, providing in some cases divergent findings. **Methods:** The lipid profile changes in response to 1-year treatment with different statins in 378 dyslipidemic patients (189 men and 189 women, aged 20-82 years) were evaluated. **Results:** In this large series of patients, a significantly attenuated reduction of total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels in women compared to men upon drug treatment was noted. A gender difference in the reduction of LDL-C was also noted according to baseline high-density lipoprotein cholesterol (HDL-C), which was particularly evident in women with baseline HDL-C > 60 mg/dl (-22.1% vs. -26.4%, women vs. men). **Conclusions:** The present study suggests that statin treatment seems to have a reduced effectiveness in improving the plasma lipid profile in dyslipidemic women compared to men. Whether such gender difference

may have an impact on health, leading to a lower preventive activity, remains to be elucidated.

**CVBM2. Anti-Lp(a) Antibody: For Diagnosis and Therapy**

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**Background:** The presence of the lipoprotein(a) in plasma was first described by Kare Berg in 1963 as an LDL-like particle. Lp(a) was later identified as a risk factor for atherosclerotic diseases because of its pro-atherogenic, prothrombotic and antifibrinolytic properties. Different epidemiological studies have also suggested that Lp(a) could increase the risk of cardiovascular disease and ischemic stroke if associated with other predisposing factors such as hypercholesterolemia, hypertension, diabetes mellitus and low level of HDL. There is also experimental evidence that lipoproteins have a role in degenerative diseases and not only in atherosclerosis. Today there are few therapeutic approaches for the treatment of hyperlipidemia(a). High-affinity monoclonal antibodies are an attractive therapeutic alternative. Due to their specificity, they have the ability to selectively bind the molecule of interest, and their structure, which includes an Fc region, allows complex binding to the Fc receptor localized on the surface of monocytes and macrophages.

**Methods:** In this study, we sought to characterize the effect of anti-Lp(A) monoclonal antibody 2E8, directed toward KIV2, in an *in vitro* system. The ability of the antibody to induce internalization of Lp(a) in murine macrophages (RAW cells) has been tested by an ELISA test and by microscopical evaluation of intracellular lipid accumulation.

**Results:** The number of foam cells had increased five times compared to the non-MAb control. **Conclusions:** This system will allow selection of new MABs generated against human-Lp(a), and a fine characterization of the cellular response triggered by Lp(a)-MAB complexes.

**CVBM3. Protein Array Analysis of Pro-Inflammatory Status in Patients with Different Heart Diseases**

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**Background:** Cardiovascular diseases (CVDs) have been associated with inflammation and cytokine modulation and altered levels of these have been observed in the plasma of patients with various heart diseases. Our goal was to investigate new possible markers of heart disease to better understand involvement of these in different pathology such as coronary artery disease (CAD) and cardiac valvular replacement (VR). **Methods:** We have studied 3 groups of subjects: the first consisted of 20 patient affected by CAD undergoing coronary artery bypass grafting (CABG); the second of 20 patient with no signs of CAD undergoing VR; and the third group of 20 healthy man without apparent pathologies as controls. The analytes of interest were quantified using a biochip array analyzer (Evidence, Randox Ltd., Crumlin, UK). Analytes researched in our study were: IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , TNF R1, R2 and INF- $\gamma$ . **Results:** Our data showed a statistically significant increase of plasma levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-1, TNF- $\alpha$  and INF- $\gamma$  in CABG and VR patients compared to controls ( $P < 0.05$ ). Our data also showed a statistically significant increase of IL-1 $\alpha$  and IL-6 plasma levels in CABG patients compared to VR, whereas IL-1 $\beta$ , IL-1, TNF- $\alpha$ , and INF- $\gamma$  were not different between the two groups. **Conclusions:** Our data suggest that CVD patients showed an inflammatory status due to an increase of pro-inflammatory and a reduction of anti-inflammatory mediators compared to healthy patients.

**CVBM4. The Epicardial Adipose Tissue as a Potential Source of IL-18**

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**Background:** The observation that circulating levels of IL-18 are elevated in human obesity and that weight loss is associated with proportional reduction of its level suggested that a likely site for IL-18 production may be the adipose tissue. Due to the link between obesity, inflammation and cardiovascular diseases, we aimed to measure IL-18 circulating levels in patients with different degree of adiposity undergoing open-heart surgery both to coronary artery bypass grafting (CABG) surgery or to valve replacement (VR) and we also evaluated whether epicardial adipose tissue (EAT) may be a potential source of IL-18. **Methods:** Blood samples of patients undergoing elective CABG or VR surgery and of lean control subjects were collected after an overnight fasting to measure IL-18 levels by immune-enzymatic assay. IL-18, IL-18 R1 and IL-18-RAP gene expression were evaluated on EAT biopsy harvested from CABG and VR patients. **Results:** Quantification of IL-18

protein indicated that patients had higher IL-18 level than controls, considered both together ( $303.76 \pm 132.23$  pg/mL vs.  $124.75 \pm 15.03$  pg/mL, mean  $\pm$  SD,  $P < 0.0001$ ) and after subdivision in CABG ( $282.89 \pm 79.33$  pg/mL,  $P < 0.001$ ) and VR patients ( $354.79 \pm 211.94$  pg/mL,  $P < 0.01$ ). Also after classification of the patients in subgroups according to their body mass index (BMI) (normal-weight and overweight/obese), IL-18 levels were higher than those in control group. **Conclusions:** It seems that although these two different groups of patients had similar increased circulating levels of IL-18, which were independent of the BMI status of the subjects, a different local biology for IL-18 may exist at EAT level.

#### CVBM5. Interleukin-15, Coronary Artery Disease and Epicardial Adipose Tissue: Possible Correlation

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**Background:** The epicardial adipose tissue (EAT) has been shown to increase in obesity and to play a potential role in the development of coronary artery disease (CAD) by secreting different mediators. Interleukin-15 (IL-15) is one of the cytokines expressed by the inflammatory cells located at atherosclerotic plaques. In our study we measured IL-15 plasma level in two groups of patients with different degree of adiposity: 1) patients affected by CAD and undergoing coronary artery bypass grafting surgery (CABG); 2) patients without CAD undergoing valve replacement surgery (VR). We also compared gene expression levels of IL-15 and its receptor (IL-15RA) in EAT samples isolated from CABG and VR patients. **Methods:** Blood samples of patients CABG or VR were collected after an overnight fasting to measure IL-15 level by immune-enzymatic assay. IL-15 and IL-15RA gene expression were evaluated on EAT biopsy harvested from CABG and VR patients. **Results:** IL-15 plasma level resulted higher in CABG than in VR patients ( $3.70 \pm 1.17$  pg/mL vs.  $2.52 \pm 1.04$  pg/mL; mean  $\pm$  SD;  $P < 0.05$ ). After classification according to BMI, IL-15 level resulted higher in overweight/obese (OB) CABG compared to OB VR patients ( $4.54 \pm 0.30$  pg/mL vs.  $2.18 \pm 0.52$  pg/mL;  $P < 0.01$ ). A trend of increase was also observed in normal-weight (NW) CABG compared to NW VR patients ( $3.58 \pm 0.40$  pg/mL vs.  $2.63 \pm 0.08$  pg/mL). Only in CABG group IL-15 level was higher in OB than in NW group ( $4.54 \pm 0.30$  pg/mL vs.  $3.58 \pm 0.40$  pg/mL;  $P < 0.001$ ). **Conclusions:** The increased IL-15 circulating level observed in CABG vs. VR patients seemed more correlated to the CAD pathology than to the obesity status of the patients. Whether EAT may significantly contribute to increase IL-15 circulating levels in these patients need further investigation.

#### CVBM6. Pathophysiological Implications of Inflammation and Genetic Inflammatory Factors in Hypertensive and Old Patients Affected by Sporadic Thoracic Aortic Aneurysm

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**Background:** Sporadic thoracic aortic aneurysm (S-TAA) is potentially devastating with severe morbidity and mortality. Current evidence suggests inflammation as main mechanism of its pathophysiology associated with both aging and age-related hypertension. Thus, we assessed whether inflammation is the principal mechanism of medial degeneration in hypertensive and old S-TAA. **Methods:** Histopathological and immunohistochemical aorta examination was executed. Furthermore, genotyping of ten SNPs in cases and controls was performed. Plasma inflammatory molecules were also detected in patients and controls using ELISA technique. **Results:** A significant inflammatory/immune CD3+CD4+CD8+CD68+CD20+ cellular infiltrate mainly in vasa vasorum of adventitia was observed in case aortas, suggesting its possible migration from these vessels into media and its role in destroying all components of extracellular matrix and vascular smooth muscle cells (VSCM). Consistent with these data, significant higher plasma levels of systemic inflammatory mediators characterized the cases. Different aorta abnormalities, apoptosis of VSCMs and severe MMP-9 amounts were also found in S-TAA aortas. In addition, five very significant associations with S-TAA risk were detected. Of these, D/I ACE and -1562 C/T MMP-9 SNPs are independent risk factors for S-TAA. Higher tissue and plasma levels of MMP-9 were also observed in -1562T MMP-9 allele carriers. A high S-TAA risk genotype was also detected significantly associated with high levels of systemic inflammatory mediators, immune/inflammatory cells and hypertension. **Conclusions:** Results obtained agree emerging evidence of inflammation as shared pathological mechanism for S-TAA, suggesting the role of inflammatory products and genetic profile as possible S-TAA risk biomarkers.

#### CVBM7. Role of Oxysterols in the Progression of Atherosclerotic Lesions

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**Background:** Atherosclerosis has been associated with chronic inflammation which contributes to atherosclerotic plaque progression and rupture. Matrix metalloproteinases (MMP), secreted mainly by macrophages, play a major role in the extracellular matrix remodeling. Dysregulation between MMP and tissue inhibitors of metalloproteinases (TIMP) can render the plaque vulnerable. Increased MMP-9 expression has been found in atherosclerotic plaques of patients experiencing cardiovascular diseases. An association between oxidized LDL and coronary heart disease has also been demonstrated. Oxysterols, cholesterol oxidation products which are abundant in oxidized LDL, appear to be involved in the pathogenesis of atherosclerosis. **Methods:** Human promonocytic U937 cells were treated with an oxysterol mixture, whose composition is similar to that found in human plaques. MMP-9, TIMP-1/TIMP-2 expression was measured by real time RT-PCR whereas MMP-9 and TIMP protein levels were analyzed by Western blotting; MMP-9 enzymatic activity was analyzed by zymography. The production of reactive oxygen species (ROS) was observed by confocal microscope. **Results:** Our results show that the oxysterols induce a significant increase of expression, synthesis and enzymatic activity of MMP-9, whereas they don't affect the expression and synthesis of the inhibitors TIMP-1/TIMP-2. Using inhibitors or specific siRNAs, we demonstrated that oxysterols induce MMP-9 expression through: 1) PKC-mediated NADPH oxidase- and mitochondria-dependent ROS overproduction; 2) up-regulation of ERK1/2 and JNK signaling pathways; 3) up-regulation of AP-1- and NF- $\kappa$ B-DNA binding. Moreover, oxysterols induced inflammatory cytokine expression which might contribute to plaque vulnerability by inducing MMP-9 expression. **Conclusions:** In conclusion, oxysterols significantly contribute to the plaque vulnerability by promoting MMP-9/TIMP-1/2 imbalance in phagocytic cells.

#### CELL DEATH PATHWAYS

##### CDP1. Magnesium and Its Mitochondrial-Specific Channel (mrs2) in Doxorubicin-Induced Apoptosis of Mammary Epithelial Cells

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**Background:** Several apoptotic stimuli induce a magnesium release from mitochondria that seems to be functional to the apoptotic process. Mitochondrial RNA splicing gene 2 (*mrs2*) transcribes a channel protein, homologous to the bacterial CorA, which mediates a membrane potential-driven magnesium uptake into mitochondria. Interestingly, *mrs2* expression has been associated to tumour multidrug resistant phenotype, although the mechanism remains unclear. We here investigated the role of *mrs2* in doxorubicin-induced apoptosis in mammary epithelial cells. **Methods:** Mammary epithelial cells (HC11) were adapted to grow in low or high magnesium medium. Control HC11, high-Mg and low-Mg HC11 were assessed for sensitivity to doxorubicin-induced apoptosis (annexin-fitc, mitochondrial membrane potential and cytochrome c release) and *mrs2* expression (Western blot). *mrs2*-siRNA cells were assessed for doxorubicin sensitivity and compared to wild type counterparts. **Results:** Our results show that sensitivity to doxorubicin depends on magnesium availability. High-Mg cells and magnesium-supplemented HC11 cells (10mM for 48h) are more resistant to doxorubicin-induced apoptosis. Interestingly, in both cell lines mitochondrial *mrs2* protein was up-regulated compared to control or untreated cells. Silencing of the *mrs2* gene enhanced doxorubicin-induced apoptosis in all cells. **Conclusions:** Our data suggest that increased magnesium availability protects HC11 cells from doxo-induced apoptosis. The expression of the mitochondrial protein *mrs2* is involved in apoptosis resistance as *mrs2*-siRNA increased doxo sensitivity also in high-magnesium cells. Since *mrs2* overexpression has been associated to multidrug resistance (Chen, 2009) we hypothesize that *mrs2* and the associated mitochondrial magnesium uptake have a crucial role in the mechanism of drug resistance.

#### ENDOCRINE AND METABOLIC DISORDERS

##### EMD1. Novel Mutations in SAR1B and MTP Genes in Children with Chylomicron Retention Disease and Abetalipoproteinemia

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**Background:** Monogenic hypobetalipoproteinemias (mHBLs) include Familial Hypobetalipoproteinemia (FHBL) with a dominant transmission and

Abetalipoproteinemia (ABL) and Chylomicron Retention Disease (CMRD) with a recessive transmission. We investigated three children born from consanguineous parents, presenting with hypobetalipoproteinemia associated with chronic diarrhea and retarded growth. **Methods:** We resequenced the candidate genes for mHBLs and performed *in vitro* functional studies of mutant alleles. **Results:** Patient HBL-108 had a moderate hypobetalipoproteinemia, apparently transmitted as dominant trait, suggesting the diagnosis of FHBL. However, she had no mutations in FHBL candidate genes (*APOB*, *PCSK9* and *ANGPTL3*) nor in *MTPP* gene (ABL). She was found to be homozygous for a novel mutation in *SAR1B* gene resulting in a missense mutation (p.Glu62Lys), as a possible cause of CMRD. In patients HBL-103 and HBL-148 the severity of hypobetalipoproteinemia and its recessive transmission suggested the diagnosis of ABL. The *MTPP* gene sequencing showed that these patients were homozygous for a nucleotide substitution in the donor splice site of intron 9 (c.1236 +2 T>G) (patient HBL-103) and intron 16 (c.2342+1G>A) (patient HBL-148) predicted *in silico* to obliterate the splice site. *In vitro* assay with splicing mutation reporter *MTPP* minigenes showed that intron 9 mutation caused the skipping of exon 10, whereas intron 16 mutation caused a partial retention of this intron in the mature mRNA. The products of these mRNAs are truncated proteins devoid of function. **Conclusions:** The diagnosis of the rare disorders ABL and CMRD should be considered in children born from consanguineous parents presenting with chronic diarrhea associated with hypobetalipoproteinemia.

#### EMD2. Oxidative Stress During Prolonged Exercise in Insulin-Dependent Type 1 Diabetic Patients

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**Background:** Several studies showed that diabetes mellitus (DM) is accompanied by increased formation of free radicals and decreased antioxidant capacity, leading to oxidative stress. Physical activity is part of the management of DM; however, the impact of exercise on oxidative stress is unclear. We aimed at investigating the oxidative stress during prolonged moderate exercise in a group of type 1 DM patients and a group of well-matched healthy controls. **Methods:** Nine patients (47 ± 10 years, 73 ± 15 kg weight, 170 ± 10 cm stature; Hba1c 7.1 ± 1.1%) and 15 healthy controls (46 ± 10 years, 75 ± 16 kg weight, 174 ± 10 cm stature) performed a 3-hrs constant intensity walk at 30% of the heart rate reserve. Patients were administered appropriate amounts of carbohydrates to avoid an excessive fall of glycemia. Venous blood samples were obtained before and at the very end of the trials for determination of glucose and insulin levels. Capillary blood samples were taken at the start of the walks and thereafter every 30 min to perform the Free Oxygen Radicals Test (FORT, CR-2000 Callegari1930, Italy). **Results:** Glucose and insulin levels were higher in patients than in controls. Type 1 DM patients showed higher oxidative stress values as compared to healthy controls (380.1 ± 14.7 vs 293.1 ± 9.6 arbitrary units; *P* < 0.05). Nevertheless, oxidative stress remained constant in both groups of volunteers throughout the whole exercise (*P* = NS). **Conclusions:** The illustrated data show that, even if type 1 diabetic patients show higher oxidative stress values as compared to healthy people, prolonged moderate exercise does not exacerbate this potentially harmful condition.

#### EMD3. Preliminary Evidence of a Peculiar Hormonal Profile in Men with Adverse Effects After Use of Finasteride Against Androgenetic Alopecia

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**Background:** Finasteride is a 5- $\alpha$ -reductase inhibitor that impairs the conversion of testosterone (T) to dihydrotestosterone (DHT). At dosage of 1 mg/die finasteride is successfully used against androgenetic alopecia. In young men finasteride used against hair loss is reported to provoke reversible sexual side effects. However, some very recent reports highlighted long-term persistence of sexual dysfunctions. We aimed to hormonally characterize 9 patients with long-term post-finasteride syndrome. **Methods:** Nine patients (36 ± 5 years old) with persistent (over 6 months) adverse effects including erectile dysfunction, infertility and depression, and 10 healthy matched controls were enrolled. Testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), progesterone and prolactin levels were evaluated in morning serum of all subjects. **Results:** Testosterone concentrations did not differ in cases and controls, *P* = 0.74. However, LH was 2-fold lower in cases (*P* = 0.03), whereas FSH was not statistically different between groups. Interestingly, the ratio of T/LH was 1.8-fold higher in cases than in healthy controls (*P* = 0.04). **Conclusions:** A concerning evidence is accumulating on severe long-term consequences of finasteride use in less than 50 years-old men. The percentage of subjects having post-finasteride syndrome is still to be determined and reasons of such persistent effects are unknown. We were the first to determine a peculiar hormonal profile in these patients suggesting an impairment of the

endocrine interplay of hypothalamus, pituitary and the testis, which specifically dampens only one of the gonadotrophins released from the pituitary gland, i. e. LH.

#### EMD4. Leptin-Induced mTOR Activation Defines a Specific Molecular and Transcriptional Signature Controlling CD4+ Effector T Cell Responses

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**Background:** The sensing of T cells of metabolic and energetic changes in the microenvironment can determine the differentiation, maturation and activation of these cells. Although it is known that the mammalian target of rapamycin (mTOR) gauges nutritional and energetic signals in the extracellular milieu, it is not known how mTOR and metabolism influence CD4+CD25-FoxP3- effector T cell (Teff) responses. **Methods:** We characterized at cellular, biochemical and transcriptional level, the effect of leptin on Teff, both *in vitro* and *in vivo*. **Results:** Here we show that leptin-induced activation of mTOR, which in turn controls leptin production and signalling, causes a defined cellular, biochemical and transcriptional signature that determines the outcome of Teff responses, both *in vitro* and *in vivo*. The blockade of the leptin/leptin receptor signalling, induced by genetic means or by starvation, leads to impaired mTOR activity that inhibits *in vivo* the proliferation of Teff. Notably, the transcriptional signature of Teff cells in the presence of leptin blockade appears similar to that observed in rapamycin-treated Teff. **Conclusions:** These results identify a novel link between nutritional status and Teff responses through the leptin-mTOR axis, and define a potential target for Teff modulation in normal and pathologic conditions.

#### EMD5. A Metabolomic Approach to Selective Intrauterine Growth Restriction (sIUGR) in Monochorionic Pregnancies

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**Background:** The aim of the present study was (1) to compare abdominal aorta intima-media thickness (aIMT) among selective intrauterine growth restricted (sIUGR) monochorionic (MC) twin fetuses with and without Doppler umbilical artery (UA) vasculopathy and AGA twin fetuses in utero, (2) to compare metabolomic profiles in MC sIUGR twins among the groups. **Methods:** 26 MC diamniotic (DA) twins pregnancies with and without sIUGR were included in the study. sIUGR in MC pregnancies was defined as an estimated fetal weight < the 10th percentile in the smaller twin. sIUGR twins were further divided in two groups based on abnormal UA Doppler waveforms. aIMT was determined for each twin at 32 weeks. Fetal blood samples were obtained from the umbilical vein from a doubly clamped segment of each cord immediately after fetal extraction. The metabolomic analysis was performed comparing the LC-HRMS spectra obtained with the three different groups of twin pregnancies. **Results:** MC twins were divided in three groups in base of sIUGR and Doppler UA waveforms. In the sIUGR MC twins presenting abnormalities of UA Doppler velocimetry aIMT was higher than in the AGA one. There was also an elevation of phenylalanine, tryptophan, sphingosine and glycerophosphocoline levels in this group. **Conclusions:** aIMT is higher in sIUGR twins with Doppler velocimetry anomalies. An elevation of phenylalanine, tryptophan, sphingosine and glycerophosphocoline levels in the first group could represent in part the clinical sign of endothelium dysfunction in fetuses with a low birth weight.

#### HEMATOLOGY

##### HEM1. Diagnostic Efficiency Improvement in Onco-Hematology Diseases Using Selected Cell Population

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**Background:** In chronic myeloproliferative neoplasms (MPNs), Philadelphia negative and multiple myeloma (MM), the choice of tissues to put through diagnosis/prognosis tests of molecular biology and molecular cytogenetics is of utmost importance.

**Methods:** We wanted to confirm the improvement of the diagnostic efficiency by effecting molecular tests on the cell lines involved in the onset of the pathology, the granulocytes in the MPNs and the plasma cells of the MM against whole blood and bone marrow. The researched molecular markers are the V617F of the *JAK2* gene within the MPNs, the deletion of the *P53* gene, rearrangements of the *IGH@* gene and the D13S25-D13S319 markers for MM. **Results:** The evaluation of the V617F mutation of *JAK2* was performed on 110 samples, amplifying through Real-Time PCR both for the DNA extracted from whole blood and isolated granulocytes. We

identified two mutated samples only on the DNA extracted from the granulocytes. A selection of 10 cases of MM presenting one of the researched genetic anomalies was made. The percentage of anomalous clones encountered within the plasma cells was always higher than the one observed in the bone marrow, but especially identifiable in the first 100 analysed cells. However, 300 to 5,000 cells should be analysed to observe the anomalous clone in the bone marrow. **Conclusions:** The selection of the cellular population on which to perform specific tests was essential to improve the diagnostic efficiency, either in terms of sensibility and in the precocity of the diagnosis.

#### HEM2. Splenectomy Is a Risk Factor for Developing Hyperuricaemia and Nephrolithiasis in Thalassaemia Intermedia Patients: A Retrospective Study

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**Background:** In patients with thalassaemia major and intermedia, hyperuricaemia and nephrolithiasis have been already described; however, reliable data about their occurrence, pathophysiological basis and consequences are lacking. **Methods:** We retrospectively reviewed the charts and radiological studies of 89 patients with thalassaemia intermedia followed at our clinic with routine biochemical examination and radiological imaging of the urinary tract with the aim to analyze both the prevalence of hyperuricaemia and nephrolithiasis and their impact on renal function. **Results:** Renal calculi were identified in 11 patients (12%) and 22 patients (25%) were under uricosuric treatment for hyperuricemia. The prevalence of nephrolithiasis increased with age but not in a statistically significant manner. Major risk factors for renal stone formation were splenectomy (in 91% of the cases, O.R. =13.6) and higher number of erythroblasts. Patients with renal stones had lower GFR value and had significantly higher level of urate with respect to those observed in patients not affected. Stone formers had higher mean creatinine level and lower GFR value with respect to those observed in patients without urolithiasis although the difference was nearly statistically significant ( $P = 0.051$ ) only for creatinine level. **Conclusions:** Our data suggest that splenectomy, by further increasing erythrocyte turnover and number, may be directly involved in the pathogenesis of hyperuricemia and nephrolithiasis observed in thalassaemia intermedia patients; our findings attributed additional disease-related complications to age and to splenectomy in thalassaemia intermedia patients.

#### HEM3. Endogenous Thrombin Potential in Thrombophilic and Non-Thrombophilic Women with History of Complications During Pregnancy

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**Background:** Thrombophilia increases the risk of complications during pregnancy: IUGR, miscarriage, MEF, eclampsia. These complications can be present in healthy women too, and the hypercoagulable state, not detected in common tests, has been invoked as an alternative cause. Endogenous Thrombin Potential (ETP) may constitute a valuable diagnostic aid in defining a hypercoagulable condition. **Methods:** Out of the 84 women with complications, 41 were thrombophilic and 43 were not. Thrombophilic condition showed: 7 x FV Leiden, 11 x F II, 15 with ACA, 1 x LA, 1 x Protein S deficit, 1 x Protein C deficit, 3 x FV + FII, 1 x FV + hyperhomocysteinemia, 1 x ACA + Protein S deficit. In thrombophilic patients the complications were: 2 women with eclampsia, 33 with more miscarriages, 1 IUGR, 4 MEF, 1 premature delivery; in patients with no thrombophilia: 9 women with eclampsia, 17 with more miscarriages, 4 IUGR, 8 MEF, 5 premature delivery. 36 healthy women with normal pregnancy were used as control. No patient in this study had cardiovascular risk or took any antithrombotic medication. ETP was measured by the chromogenic method (SIEMENS) on platelet-poor plasma. **Results:** ETP evaluation did not show significant differences between thrombophilic women (median 93% (range 74%-130%)) and non-thrombophilic women [median 97% (range 71%-124%)]. ETP in the control group is comparable to the two populations studied [median 108.3% (range 80.9%-127.9%)]. **Conclusions:** ETP shows no correlation between thrombophilia and complications in pregnancy.

#### HEM4. Pretest Clinical Score (4Ts) and Laboratory Testing for Reliable Diagnosis of Heparin-Induced Thrombocytopenia

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**Background:** Heparin-induced thrombocytopenia (HIT) is an uncommon but potentially devastating complication of anticoagulation with unfractionated heparin

(UHF) or low molecular weight heparin (LMWH). HIT is defined as a decrease in platelets to less than 50% or to less than 100 x 10<sup>9</sup>/L and positive laboratory HIT assay. Early diagnosis of HIT leads to rapid interruption of the eparinic treatment, substituted by alternative anticoagulant therapies, reducing mortality from 23 to 1.1%. When HIT is suspected (score 4Ts) rapid and highly sensitive diagnostic tests are required. ELISA and functional cytofluorimetric tests are highly specific and sensitive, but are time- and labor-consuming. **Methods:** We re-evaluated 61 samples from patients from 2011, showing high or moderate HIT, as assessed by 4Ts clinic test. The turbidimetric immunological test (Hemosil HIT-Ab-IL) was performed on all of them and showed the presence of anti-PF4/Eparine antibodies in 6 patients (9.8%). These samples were subsequently functionally tested at the cytofluorometer and by chemiluminescence (Hemosil AcuStar-IL). **Results:** Both tests were positive in only 3 patients (4.9%); there was a strong correlation between the chemiluminescent immunological test and the functional cytofluorimetric one. **Conclusions:** The chemiluminescence test, being as sensitive and specific as the cytofluorimetric one, but easier and faster, might represent the elective test for HIT diagnosis. However, this test is suitable only for patients who scored high or moderate for HIT at the clinical test.

#### HEM5. Bioinformatics as a Starting Point for the Analysis of ALK1 Missense Mutations

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**Background:** Activin A receptor, type II-like kinase 1 (also called ALK1), is a serine-threonine kinase predominantly expressed on endothelial cell surface, involved in TGF- $\beta$  signalling. It is encoded by the *ACVRL1* gene (12q11-14), whose mutations cause type 2 Hereditary Hemorrhagic Telangiectasia (HHT2), an autosomal dominant multisystem vascular dysplasia. Although an X-ray structure of ALK1 intracellular domain has recently become available (PDB ID: 3MY0), structure determination of ALK1 ectodomain (ALK1EC) has been elusive so far. **Methods:** We recently described the building of a homology model for ALK1EC, followed by an extensive bioinformatic analysis, based on a set of 38 methods, of the effect of missense mutations at the sequence and structural level. ALK1EC potential interaction with its ligand BMP9 was then predicted combining modelling and docking data. **Results:** Major structural changes and loss of stability of the protein were predicted for several mutations, whereas others were found to interfere mainly with binding to BMP9 or other interactors, like Endoglin (CD105), whose encoding *ENG* gene (9q34) mutations are known to cause type 1 HHT. **Conclusions:** Building on these predictions, we are now creating a library with the most interesting mutations, both by gene synthesis and site-directed mutagenesis, to try to express them and analyse their effects on endothelial cells by *in vitro* and *in vivo* tests, as well as to determine the real crystal structures.

### IMMUNITY AND INFLAMMATION – AUTOIMMUNE DISEASES

#### IAD1. Advances in Anti-Topoisomerase I Antibodies Evaluation

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**Background:** Positivity of Anti-topoisomerase I (anti-topo I) antibodies in systemic sclerosis is historically correlated with disease activity and severity. On the contrary, a small number of studies are focused on the "quantity" of these antibodies. Our study aims at evaluating the relations between the "quantity" of antibody and the activity/severity of the disease. **Methods:** In 30 scleroderma patients, anti-topo I antibodies were consecutively measured in an observation time from 2-6 years. Naifold videocapillaroscopy was made at baseline. Clinical evaluation was performed by Eustar score for disease activity and Medsger scale for disease severity. Anti-topo I were tested by EliA technology (Phadia, Germany). **Results:** For each patient we calculated the median value of anti-topo I. Median values were categorised in 3 groups: lower tertile 164 U/ml. Eustar evaluation: the median values of anti-topo I showed a positive correlation with Eustar score and a significant difference emerged in the patients of the lower tertile in comparison with the upper tertile. Medsger evaluation: in presence of low levels of anti-topo I the organ-system involvement is often absent or at least limited. Also videocapillaroscopy findings agree with anti-topo I levels. **Conclusions:** Our data show differences mostly between low and high levels of anti-topo I. Despite this limitation a routinely quantitative expression of anti-topo I results seems to be a useful tool for clinical evaluation until the first observation at diagnosis.

**IAD2. Serum Matrix Metalloproteinase-3 Assessment in Rheumatoid Arthritis: Two Methods Compared**

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**Background:** Several recent papers highlighted the possible role of serum matrix metalloproteinase-3 (MMP-3) assessment in rheumatoid arthritis (RA) as a reliable tool in the diagnosis, prognosis and to detect therapeutic efficacy (Houseman, Arthritis Res Ther 2012). Here we tested 2 different ELISA methods to analyse MMP-3 serum levels in routinely attending patients with rheumatoid arthritis.

**Methods:** 23 RA patients were enrolled (17 females, age range 33-79), tested more times in the follow-up, total samples n. 95. 42 age and sex-matched healthy donors (HDs - 27 females, age range 20-64). MMP-3 serum levels were analysed by two ELISA kits: DiaMetra MMP-3 ELISA (DiaMetra, Segrate, Milano, Italy) and AESKULISA MMP-3 (AESKU.diagnostics, Germany). **Results:** As expected, MMP-3 serum levels in HDs were significantly more elevated in males than in females, as assessed by both methods, even with slightly different ranges (25.4 ± 5 ng/ml vs 12.7 ± 3.4 ng/ml by DiaMetra; 86 ± 24.2 ng/ml vs 36.3 ± 10.5 ng/ml by AESKU). RA patients generally disclosed higher levels than HDs, either by DiaMetra (45.5 ± 39 ng/ml) and by AESKU (200 ± 203 ng/ml) and a good correlation between serum MMP-3 and disease activity was found in the follow-ups. A very good correlation was found between the results obtained in the same sera by the two methods (Spearman r 0.97, 95%CI 0.95-0.98, P < 0.0001). **Conclusions:** Both the MMP-3 ELISA methods work well in the assessment of MMP-3 serum levels in routinely attending RA patients and this test may be useful to better characterize disease outcome in the follow-up.

**IAD3. A Single Non-Synonymous Polymorphism of TLR2 Is responsible for Variability of Experimental Multiple Sclerosis in SJL and B6 Mice**

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**Background:** Multiple sclerosis is characterized by variability of course and lesions distribution. The complexity of its presentation may reflect differences in either environment or genetic. In rodents challenge with peptides of myelin drives distinct forms of EAE in each strain/peptide pair, but it is difficult to assess the relative role of genetic background and antigens in determining the course of the disease. In all EAE models the administration of mycobacterium-derived motives is essential for disease development. TLR2 is the main receptor recognizing motives from M tuberculosis. **Methods:** To study the contribution of TLR2 genetics to EAE, we crossed SJL (TLR2 83Ile) and B6 (TLR2 83Met) mice, generating F1 of SJLxB6wt (heterozygous for TLR2 Ile83Met) and F1 of SJLxB6tlr2- (TLR2 83Ile). We then immunized both groups of F1 mice with PLP139-151 and examined course and lesion distribution of EAE. **Results:** TLR2 83Ile increases secretion of IFN-γ (P = 0.043) and IL-17 (P = 0.041), whereas IL-13 and FoxP3 are similar in both groups. Consequently there are significant differences in the EAE. F1 mice of SJLxB6tlr2- display a more severe EAE (P = 0.0004) in the absence of PTx administration. SJLxB6wt mice are sensitive to PTx administration, whereas F1 mice of SJLxB6tlr2 are not. EAE developed in SJLxB6wt mice has a clear progressive/chronic clinical course, whereas that obtained in SJLxB6tlr2- mice often show incomplete and of short duration signs of remission. SJLxB6tlr2- mice frequently display inflammatory lesions and demyelination in the frontal lobes. **Conclusions:** Thus, a single polymorphism of TLR2 modifies significantly clinical and histology of EAE.

**IAD4. Phenotypic Characterization of Circulating B- and NK-Cell Subsets as a Marker Of Primary Sjögren's Syndrome (pSS)**

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**Background:** Primary Sjögren's syndrome (pSS) is an autoimmune disorder characterized by chronic inflammation and loss of function of salivary/lachrymal glands. pSS patients show increased circulating levels of BlyS, able to prevent B cell apoptosis and increase NK cell functions. Here, we characterize the phenotype of B and NK cells in pSS. **Methods:** 14 pSS patients fulfilling the new American-European Consensus Group diagnostic criteria and 14 age/sex-matched healthy controls were studied. PBMCs were analyzed through an 8-colors flow-cytometry approach and the serum level of B-Lymphocyte Stimulator (BlyS) was assessed by

ELISA. **Results:** All pSS patients were SSA/SSB-positive, 12 were RF-positive and they had higher BlyS serum levels. Histologically, 8 patients presented MESA and 4 had a history of MALT-type lymphoma in the parotid glands. In the pre-immune B-cell compartment, patients showed a significant reduction of marginal-zone-like B cells (CD27+ IgD+ IgM+) compared to controls (2.96 ± 2.15% vs 4.75 ± 1.89%; \*P = 0.0328) and an increased percentage of naive B cells (CD27- IgD+ IgM+) (70.76 ± 14.40% vs 60.36 ± 7.63%; \*P = 0.0126). In the antigen-experienced B-cell compartment, patients exhibited a significant reduction of the IgM-only memory (CD27+ IgD- IgM+, 0.62 ± 0.77% vs 2.01 ± 1.05%; \*P = 0.0013), switched-memory (2.52 ± 2.54% vs 7.90 ± 4.28%; \*\*\*P = 0.0003) and CD27- memory (7.85 ± 5.49% vs 11.82 ± 5.49%; \*P = 0.037) subpopulations. CD56+/CD16+ NK cells were significantly reduced in patients (11.20 ± 17.54% vs 16.29 ± 7.86; \*P = 0.0224), as well as the CD56-/CD16+ NK cells (0.73 ± 1.67% vs 0.78 ± 0.45; \*P = 0.0192).

**Conclusions:** In summary, pSS patients showed significant alterations in circulating B- and NK-cell compartments, possibly associated with the high levels of serum BlyS and aberrancies of cell homeostasis at tissue gland level.

**IAD5. Clinical and Serological Response to Tocilizumab in Rheumatoid Arthritis Patients**

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**Background:** Rheumatoid factor (RF) IgM and antibodies to citrullinated proteins (ACPA) are serological markers of rheumatoid arthritis (RA), currently assessed in clinical practice. Yet the measurement of IgG- and IgA-RF is not performed routinely because of conflicting data on the clinical relevance of these isotypes. Data confirming a definite relationship between decreased RF levels and clinical response are scarce. The aim of the present observational longitudinal study was to investigate whether RF isotypes and ACPA are related to clinical response in RA patients treated with tocilizumab (TCZ, anti-IL6R). **Methods:** The study population was composed by 27 subjects (24 females, mean age 56.4 ± 10.7 years). The subjects were studied at baseline (T0), and at follow up visits at 3 (T1) 6 (T2), and 12 months (T3) after the beginning of the treatment with TCZ 8 mg/Kg. Each patient was assessed at each time point through clinical scales (VES, PCR, HAQ, DAS28-VES, DAS28-PCR, CDAI, and SDAI). IgM-, IgA- and IgG-RFs and anti-CCP antibodies were assessed by enzyme linked immunosorbent assay at T0, T1, T2, T3. **Results:** All patients showed a rapid, significant, and sustained clinical response to treatment throughout the observation period. Whereas the clinical scales (except HAQ) significantly decrease during time, the antibody counts do not. We only found a significant correlation (P = 0.03) between ACPA and SDAI changes from baseline at T1 and T2. We found no significant correlation between the antibodies count at T0 and the changes of the DAS-28 VES at T1 and at T2. **Conclusions:** Tocilizumab, although effective in treating RA, does not decrease antibody levels.

**IAD6. Proliferative Potential of FoxP3+ Regulatory T Cells in Multiple Sclerosis Patients**

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**Background:** In autoimmune diseases there are no reliable markers able to predict either loss of self-antigen tolerance or clinical progression of pathology. We investigated the relationship between the proliferative capacity of regulatory T cells (Treg), a cellular subset that controls the immune response, with clinical progression in multiple sclerosis (MS). **Methods:** Since proliferation of Treg cells is inhibited by the adipocytokine leptin we investigated the capacity of Treg cells to expand upon leptin-neutralization *in vitro* and correlated this phenomenon with the clinical stage/progression of disease. **Results:** Purified Treg cells from MS patients, showed a reduced proliferative capacity during anti-CD3CD28-stimulation upon leptin-neutralization when compared with healthy controls. Interestingly the patients clinical state inversely correlated with the *in vitro* expanding capacity and proliferative potential of Treg cells. Indeed, patients with a higher Expanded Disability Status Scale (EDSS) showed a reduced expansion of Treg cells upon leptin neutralization. We applied the multinomial logistic model to calculate the relative risk for MS patients to develop a worst clinical progression of pathology in function of the observed expansion index at diagnosis. Thanks to this model we found a higher risk to develop a more severe MS in patients with a lower expansion of Treg cells.

**Conclusions:** Our findings could be of relevance in understanding the pathogenesis

of MS and introduce the use of the *in vitro* Tregs expansion index as marker for evaluation of immunological tolerance and disease progression in MS patients.

**IAD7. Meta-immunological Profile of Children with Type 1 Diabetes: Toward the Possibility to Predict Progression of Autoimmune Diabetes**  
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**Background:** Type 1 Diabetes (T1D) is an autoimmune disease characterized by a T cell-mediated destruction of pancreatic  $\beta$ -cells. The time course and the precise mechanisms of progressive  $\beta$ -cells failure are still not completely elucidated.

**Methods:** Nine circulating biological compounds, at the interface between immune and metabolic regulation, and twenty immune cell populations were investigated over time as markers associated with disease progression at different disease stages (high-risk subjects, T1D at onset, 12 and 24 months after diagnosis). The correlation matrix among the different biological parameters was statistically evaluated and visually assessed on 2-dimensional graphs. Finally, we built a multivariate logistic regression model to identify markers able to predict residual  $\beta$ -cell function. **Results:** We observed that the meta-immunology profile significantly differed among the different study groups and during disease progression, thus defining a specific signature typical of disease progression. Further, we defined a simple and robust decision rule, based on the multivariate logistic regression model, by measuring the number of circulating CD3+CD16+CD56+ cells and the percentage of myeloid Dendritic Cells (mDCs) at disease onset. This model was able to predict pancreatic residual C-peptide production by  $\beta$ -cells up to one year after disease onset.

**Conclusions:** This study defines a specific meta-immunology asset in T1D changing during disease progression and provides a simple decision rule that predicts residual  $\beta$ -cell function and monitors the meta-immunology status typical of T1D.

#### IMMUNITY AND INFLAMMATION – HOST DEFENSE

**IHD1. Hepatitis B Virus Isolates of Different Genotype May Vary in Their Capacity to Limit Effectiveness of Endogenous and Therapeutic Interferon- $\alpha$**   
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**Background:** Several clinical studies suggested that Hepatitis B Virus (HBV) genotype may affect response to IFN $\alpha$ -treatment of chronic hepatitis B (CHB). Aims of our study were: (a) To investigate and compare *in vitro* the transcriptional and replicative capacity of three wild-type HBV isolates of genotype A, C, and D, respectively; (b) to verify whether these three HBV genotypes display different IFN $\alpha$ -mediated induction of antiviral defence mechanisms in human hepatoma cells.

**Methods:** HBV genomes isolated from three naïve CHB patients infected with HBV of genotype A, C, and D, respectively, were full-length amplified and cloned in accordance with the method described by Gunther et al (J Virol 1995). Comparison among their transcriptional/replicative activities (Southern and Northern blot, real time-PCR and ELISA assay), recruitment of Stat1/phospho-Stat1, Stat2/phospho-Stat2 onto the HBVcccDNA (cccDNA-ChIP) and expression profile of innate immune response genes (TaqMan low-density array) were assessed after transfection of untreated- and IFN $\alpha$ -treated HepG2 cells. **Results:** Genotype A isolate produced higher levels of replicative intermediates and showed a better response to IFN $\alpha$  treatment than genotype C and D ones. Both genotype C and D HBVcccDNAs showed a reduced binding of Stat1/2 and phospho-Stat1-Stat2 compared to genotype A both in untreated and IFN $\alpha$ -treated cells. The three genotypes were able to impair TLR7, TLR9 and IRF7 expression in IFN $\alpha$ -treated cells. These three factors were down-regulated to a greater extent both in genotype C- and D-replicating cells.

**Conclusions:** Our data indicate that each HBV genotype may possess different phenotypic and biological characteristics and may differently impair pathways of the interferon response.

**IHD2. Hepatitis B Virus Causes Epigenetic Induction of Interleukin-8 Production which in Turn Inhibits Interferon- $\alpha$  Antiviral Activity**  
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**Background:** Serum interleukin-8 (IL-8) concentrations are increased in chronic hepatitis B (CHB) patients during hepatic flares. Aims of this study were: (1) to determine IL-8 amounts in sera and liver tissues of CHB patients, inactive HBV carriers (IBC) and healthy controls (HC), (2) to analyse the mechanisms implicated in HBV-induced IL-8 gene expression, (3) to study the possible antagonistic activity of

IL-8 on INF $\alpha$ -treatment response. **Methods:** Serum IL-8 amounts were analysed by an ELISA assay. IL-8 mRNA quantification in liver tissues and HBV-replicating cells was performed by real-time PCR. An IL-8 promoter-driven luciferase assay was used to study IL-8 promoter transactivation. The ChIP-assay was applied to analyse IL-8 promoter acetylation/methylation status. A cell-based HBV replication system was used to test the potential antagonistic effect of IL-8 on INF $\alpha$ -treatment response.

**Results:** CHB patients had higher amounts of IL-8 both in serum and liver tissue compared to controls. In HBV-replicating cells, IL-8 promoter transcriptional activity was stimulated up to 100-fold and IL-8 transcription was significantly increased. The luciferase-reporter-assay showed that NFKB and AP-1 are essential for IL-8 induction by HBV. HBx viral protein was recruited onto the IL-8 promoter. Acetylation/methylation status of IL-8 promoter was strongly correlated with IL-8 amounts both in serum and liver tissue. Inhibition of IL-8 by anti-IL8 monoclonal antibodies or IL-8-siRNA increased IFN- $\alpha$  inhibitory action on HBV replication in transfected cells. **Conclusions:** (1) Highly replicating HBV is able to induce IL-8 transcription by targeting the epigenetic regulation of IL-8 promoter. (2) The induction of IL-8 by HBV inhibits the INF $\alpha$  antiviral activity.

**IHD3. Local Interleukin-1 beta (IL-1 $\beta$ ) Levels in Early Pregnancy and Preterm Birth**

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**Background:** Preterm birth (PTB) is considered a research priority. The rate of PTB has not declined in the last decades. Non-invasive early biomarkers for PTB risk are highly warranted to possibly perform a personalized treatment. **Methods:** To assess if vaginal interleukin-(IL)-1 beta (IL-1 $\beta$ ) in early pregnancy is associated with adverse outcome among bacterial vaginosis (BV)-positive women, 1,806 women were enrolled at <20 weeks' gestation in 5 Philadelphia Hospitals (Philadelphia, PA). 800 women were BV-positive (by Gram evaluation according to Nugent score 7-10), 707 of them had birth outcome data. Vaginal IL-1 $\beta$  concentrations were measured in 105 BV-positive women who had an adverse preterm outcome, including 66 preterm births (20-<37 weeks, of which 52 were spontaneous) and 14 late miscarriages (12-<20 weeks), and in 295 BV controls (term normal birth weight infants). The upper (>66th percentile) and lower (<33rd percentile) tertiles of IL-1 $\beta$  concentrations were compared with the middle tertile (33rd to 66th percentile) to assess whether the risk profile is U-shaped. **Results:** None of the IL-1 $\beta$  tertiles was associated with increased risk for any adverse preterm outcome, nor preterm birth and miscarriage with or without exclusion of women with concurrent STDs. **Conclusions:** Vaginal IL-1 $\beta$  is not a risk marker for preterm birth among BV-positive women in early gestation, likely because of large overlapping of events producing actual levels of the pro-inflammatory cytokine IL-1 $\beta$  in vaginal fluid.

**IHD4. Viral N-linked Glycans Contribute to Alphavirus-induced Myositis and Arthritis**

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**Background:** Mosquito-borne alphaviruses chikungunya virus (CHIKV) and Ross River virus (RRV) cause outbreaks of debilitating infectious arthritis in humans in the Indian and Pacific Ocean regions, and CHIKV has recently become endemic in parts of Europe. CHIKV- and RRV-induced disease is characterized by severe inflammation and immunopathology within joints and muscle. Recent studies demonstrated that the mannose binding lectin (MBL) pathway of the host complement system is essential for RRV-induced immunopathology. MBL binds to terminal carbohydrates, such as mannose found on glycosylated viral proteins, to activate the complement system. There are three N-linked glycosylation sites on the RRV envelope glycoproteins that are glycosylated with high mannose and complex glycans in mammalian cells. We hypothesized that these RRV glycans are ligands for MBL binding and complement activation, contributing to development of disease. **Methods:** Using a panel of RRV mutants lacking one or more glycans, we evaluated and characterized the RRV-induced disease in mice infected with the glycan mutant viruses. **Results:** Mice infected with a virus lacking both E2 N-linked glycans exhibit reduced disease, reduced tissue damage, and reduced levels of MBL and complement activation within infected tissue compared to wild-type RRV infected mice. However, virus-induced inflammation and viral replication within infected tissues were similar between the two viruses. **Conclusions:** These results suggest that interactions between the viral N-linked glycans and MBL play a central role in the development of severe alphavirus-induced arthritis and may be an effective target for therapeutic treatment in patients infected with arthritogenic alphaviruses.

**IHD5. Mycobacterium tuberculosis in the Adjuvant Modulates Trafficking of Effector T Cells Through a Polymorphic Site of TLR2**

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**Background:** The role of infectious agents in the regulation of T cell trafficking is currently unknown. **Methods:** We examined this issue using immunoscope and TCR transgenic mice. **Results:** The amount of *M tuberculosis* in the adjuvant modulates rapid relocation of PLP139-151 (p139)-specific T cells carrying a public TCR-beta chain BV10-CASS SGS NTE JB1.1 from draining lymph nodes (LN) to spleen in the SJL mouse. In the presence of low dose of *M tuberculosis* in the adjuvant, T cells mostly reach the spleen by day 28 after immunization ("late relocation"), whereas the same T cells reach the spleen by day 14 after immunization with high dose of *M tuberculosis* ("early relocation"). The B6 background confers a dominant "early relocation" phenotype to F1 (SJL x B6) mice, allowing early relocation of T cells in the presence of low dose *M tuberculosis*. A single non-synonymous polymorphism of TLR2 (Ile83Met) is responsible for "early/late" relocation phenotype. By transferring T cells from F1 mice obtained crossing SJL mice transgenic for the TCR-beta chain indicated above (SJLBV10) with C57/B6wt or C57/B6tr2-, we determined that egress of antigen specific lymphocytes is modulated by TLR2 expressed on T cells. We also examined the expression of some markers regulated by activation and involved in T cell trafficking. Early relocation is associated with an intermediate expression of CD44 and that TLR2 also regulates processing of CD44 pre-mRNA. **Conclusions:** Pathogens engaging TLR2 on activated T cells through a polymorphic site modulate expression of activation/adhesion molecules and regulate effector T cells trafficking *in vivo*.

**IHD6. Analysis of the Polymorphisms of Th1 and Th17 Cytokines in Mediterranean Spotted Fever**

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**Background:** We have recently reported that the susceptibility for Mediterranean spotted fever (MSF) caused by *Rickettsia conorii*, is influenced by the Th2 and Th1 cytokine genetic polymorphism profiles. Less it is known on the effect of gene polymorphisms of cytokine produced by the Th17. **Methods:** 70 Sicilian patients affected by MSF and 239 control subjects matched for age, gender, and geographic origin were typed for functionally relevant single nucleotide polymorphisms (SNPs) of IFN- $\gamma$  (+874 T/A), IL-18 (-137 G/C and -607A/C) and IL-17 (7488T/C) according to our laboratory procedures. **Results:** No significant differences in IL-18 -137 G/C, -607A/C and in IFN- $\gamma$  +874 T/A genotype frequencies were observed. On the contrary a statistically significant (p value= 0.0126) increase of the IL-17 TT genotype frequency of in MSF was observed. **Conclusions:** Cytokines play a crucial role in modulation of the host defense and genetically determined differences in cytokine production seem to influence the extent and severity of a large number of infectious diseases. 7488T/C SNP impinge on IL-17 signaling and might play a crucial role in neutrophil recruitment, induction of IFN- $\gamma$  and IL-12 production in macrophages and in the induction of T regulatory cells. Our results suggest that a genetically determined increase of IL-17 dependent activation pathways might interfere with *R. Conorii* infection control.

**IHD7. The Alarmin Interleukin-33 Is a Notch Target in Quiescent Endothelial Cells**

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**Background:** The molecular mechanisms that drive expression of the alarmin interleukin-33 (IL-33) in endothelial cells are unknown. Because nuclear IL-33 is a marker of endothelial cell quiescence (corroborated in this study by showing co-expression of cyclin-dependent kinase inhibitor p27Kip1), we hypothesized that Notch signaling might be involved in regulating IL-33 expression. **Methods:** Umbilical vein-derived endothelial cells, rtPCR, immunocytochemistry, recombinant Notch ligands, siRNA, *in vivo* experiments. **Results:** Here we show that activation of Notch1 by immobilized Notch ligands was sufficient to induce nuclear IL-33 expression in cultured endothelial cells. Conversely, IL-33 expression was inhibited by the  $\gamma$ -secretase inhibitor DAPT or by inhibiting the function of Dll4, Jagged1, Notch1, or the canonical Notch transcription factor RBP-Jk. Sensitivity to cycloheximide indicated that IL-33 was a direct target of Notch signaling, well in line with the identification of several conserved RBP-Jk binding sites in the IL-33 gene. The *in vivo* expression of Dll4 but not Jagged1 was well correlated with expression of

IL-33 in quiescent vessels, and subcutaneous injection of DAPT in healthy skin reduced IL-33 expression, indicating that Notch signaling was involved. On the other hand, loss of IL-33 during angiogenesis occurred in spite of sustained Dll4 and Notch1 expression, suggesting that other signals may override the IL-33-driving signal in this context. **Conclusions:** Taken together, our data demonstrate that endothelial nuclear IL-33 is induced by Notch and that Dll4 may be the dominant ligand responsible for this signaling *in vivo*.

**IHD8. Recognition of Fungal  $\beta$ -glucan by Human Neutrophil CR3 (CD11b/CD18) Results in Homotypic Cell Aggregation and Rapid Formation of Extracellular Traps by a Mechanism That Depends on  $\alpha$ 5 $\beta$ 1**

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**Background:** The armament of neutrophil-mediated host defense against fungal and bacterial pathogens includes the extrusion of a lattice of DNA and microbicidal enzymes known as Neutrophil Extracellular Traps (NETs). The receptor-mediated interactions and intracellular signaling events responsible for elaborating NETs are not well characterized and were determined in this study for the response to *Candida albicans*. Moreover, as the host response of extravasated neutrophils to deep-seated mycotic infections necessitates contact with ECM, this study identified an important regulatory function for the ubiquitous matrix component fibronectin (Fn) in anti-fungal NET release. **Methods:** Global tyrosine phosphoproteomic analysis mitigated the quantitative analysis of phosphorylated sites of neutrophils adherent to immobilized Fn vs. Fn +  $\beta$ -glucan and was validated via Western blot analysis. Light, confocal and transmission electron microscopy was implemented to observe NET formation after addition of Sytox Green, indicating a breach in membrane integrity. **Results:** Recognition of the purified fungal cell wall pathogen associated molecular pattern  $\beta$ -glucan by human neutrophils resulted in formation of cell aggregates and NET release that required Fn. NET formation was dependent on CR3 (CD11b/CD18), but not Dectin-1 or reactive oxygen species (ROS). Fifty-four phosphopeptides were differentially regulated by  $\beta$ -glucan and validation revealed a role for ERK in aggregation and NET release. NET formation to *C. albicans* hyphae was also dependent on Fn, CR3, and ERK but not ROS. We also report a regulatory role for  $\alpha$ 5 $\beta$ 1 in mediating NET release. **Conclusions:** This study identified key receptor:ligand interactions that induce NET formation rendering insight into understanding host defense mechanisms against fungal infections.

**IHD9. Signaling Molecules Involved in Homotypic Aggregation of Human Neutrophils in Response to Fungal  $\beta$ -Glucan**

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**Background:** Complement receptor 3 (CR3), an integrin found on neutrophils, regulates firm cell adhesion to the extracellular matrix and serves as a pattern recognition receptor for the fungal Pathogen Associated Molecular Pattern (PAMP)  $\beta$ -glucan. Coincident ligation of CR3 with fibronectin and  $\beta$ -glucan is possible due to spatially distinct ligand binding domains. Current work demonstrates that dual ligation of CR3 with fibronectin at its I-domain and  $\beta$ -glucan at its lectin-like site causes homotypic aggregation of primed human neutrophils. **Methods:** Using phosphoproteomics, we have identified several proteins that are significantly phosphorylated when ligated with fibronectin and  $\beta$ -Glucan but not fibronectin alone. From these data, we propose a signaling pathway in which increased phosphorylation of phosphoinositol 3 kinase (PI3K), protein kinase C $\delta$  (PKC $\delta$ ), glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), and ERK could lead to the observed homotypic aggregation of neutrophils via CR3 on immobilized fibronectin and  $\beta$ -glucan. Western Blotting validated our initial phosphoproteomic findings for each protein and use of inhibitors obviated homotypic aggregation of neutrophils for all aforementioned proteins except for GSK3 $\beta$  in which a partial inhibition was observed. **Results:** To quantify such partial inhibition, a computer algorithm was created to help quantify the number of cells clustered in a field. With this program, we were able to determine that despite our observation GSK3 $\beta$  inhibition has no effect on cell clustering. **Conclusions:** Thus PI3K, PKC $\delta$ , and ERK are all components of the signaling pathway of dually ligated CR3 that are also relevant to homotypic aggregation.

**IMMUNITY AND INFLAMMATION – IMMUNITY**

**IMM1. Structural Basis of Affinity Maturation of Antibodies in the 2-Phenyl-5-Oxazolone System**

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**Background:** Affinity maturation of antibodies is the process whereby more efficient antibodies are produced through somatic hypermutation and antigen-guided selection. No extensive study is available at the moment concerning the relationship between somatic mutations and their structural counterpart. The antibody response to 2-phenyl-5-oxazolone has been thoroughly investigated from the genetic point of view. It consists of three antibody classes, with each member of each class derived from a unique pair of VH and VL germline genes by somatic hypermutation. In this project, we are investigating the structure of the VH and VL domains of 10 representative antibodies. **Methods:** The VH and VL domains of each antibody are being expressed as recombinant scFvs, crystallised, and their structure determined by X-ray crystallography. **Results:** The structures and models available allow an initial definition of the strategies adopted. In class I, maturation is bound to improvement of surface complementarity, especially at the top of the binding pocket, and in surface charge changes. In class II the maturation strategy seems to be based on the increase of the interacting surface, and on the introduction of a specific bond with the oxazolone ring. In class III, where the low and high affinity antibodies differ by 8 mutations, the increase in affinity is mainly determined by the improvement in the surface complementarity by removal of a bulky phenylalanine, which allows a better tightening of the two sides of the binding site. **Conclusions:** These results are relevant to determine the principles underlying affinity maturation of antibodies.

**IMM2. Identification Of CIKS/ACT1 New Interacting Partners**

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**Background:** CIKS (Connection to IKK and SAPK/JNK; a.k.a. Act1) is a putative E3 ubiquitin ligase required for signaling by IL-17. IL-17 is an inflammatory cytokine product by T helper lymphocyte type 17 (Th17), involved in the development of some inflammatory diseases like rheumatoid arthritis, psoriasis and multiple sclerosis. CIKS mediated NF- $\kappa$ B activation requires the binding to IKK $\gamma$ /NEMO (a key protein involved in NF- $\kappa$ B pathway) via the SEFIR domain; CIKS also interacts with TRAF6 (an E3 ubiquitin ligase) by its evolutionary conserved motif at the N-term. **Methods:** Immunoprecipitations of FLAG-CIKS interactors were performed with anti-FLAG antibody. The immunoprecipitation products were electrophoresed by SDS-PAGE and analyzed by mass spectrometry. **Results:** The aim of the present project is the identification of new CIKS interactors. To this purpose we infected CIKS<sup>-/-</sup> MEFs with a lentiviral expression vector containing the cDNA encoding FLAG-CIKS. The potential CIKS interactors obtained by co-immunoprecipitation were analyzed by mass spectrometry. **Conclusions:** We identified about 30 CIKS interactors whose characterization is currently under investigation.

**IMMUNITY AND INFLAMMATION – IMMUNOPATHOLOGY BIOMARKERS**

**IMBM1. Antiprothrombin/phosphatidylserine Complex Autoantibodies in Antiphospholipid Syndrome: Prevalence in Routinely Attending Patients in Udine**

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**Background:** Antiphospholipids (aPL) are a heterogeneous family of antibodies reacting with serum phospholipid-binding proteins, including prothrombin. Antibodies against prothrombin alone, or the phosphatidylserine/antiprothrombin complex may be detected. The prevalence of IgG/IgM prothrombin/phosphatidylserine complex antibodies (aPT/PS) in consecutive patients, focusing on lupus-anticoagulant (LA)-positive/anticardiolipin(aCL)-negative patients, was compared with the routinely used test for anti-prothrombin antibodies. **Methods:** 136 patients were enrolled, 78% female, age range 18-88, 109 (78.9%) LA-positive (72.4% of which aCL-negative), 24 LA/aCL-negative, 3 unknown LA, aCL-negative. Clinically: 81 lupus (SLE) or undifferentiated connective tissue diseases (UCTD), 21 with aPL syndrome (APS), 26 systemic autoimmune diseases (AID), 19 with accidental arterial and venous thrombosis (AVT), 10 LA-positive cancer-related arterial and venous thrombosis. Normal ranges were assessed on 52 healthy donors (55.8% female; age range 18-65). Quanta Lite aPS/PT IgG/IgM (Inova Diagnostics Inc, San Diego, CA). Antiprothrombin IgG/IgM (Orgentec Diagnostika, Mainz, Germany). **Results:** The overall prevalence IgG and/or IgM in LA-positive patients was in line with previous data (57.8%; Zigon, Clin Chem Lab Med 2011). The highest prevalence was found in APS (73.3%), followed by SLE/UCTD (70.4%, that were 57.4% aCL-negative) and

other systemic AID (53.8%, that were 92.3% aCL-negative). aPS/PT were positive also in 2/4 seronegative APS, in 31.6% AVT and 50% of oncologic patients. In LA-positives, the sensitivity of the new phosphatidylserine-dependent antiprothrombin ELISA was significantly higher than the anti-prothrombin alone ELISA (55.8% vs. 15.4%). **Conclusions:** Testing anti-PT/PS antibodies improves the capability to identify unrecognized aPL antigens, helping to better characterize APS patients.

**IMBM2. Laboratory Screening of *Helicobacter pylori* in Stool Samples Suggest a Relationship with Graves' Disease, but Not Hashimoto's Thyroiditis**

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**Background:** *Helicobacter pylori* (HP) infection has been epidemiologically linked to some extra-digestive conditions, including autoimmune thyroid diseases (ATDs), although there are contradictory data about relationship between HP infection and these disorders. The aim of study was to investigate the correlation between prevalence of Cag-A positive strains of HP in stool samples and ATDs. **Methods:** We investigated 112 consecutive patients: 48 females and 4 males with Graves' Disease (GD), 54 females and 6 males with Hashimoto's thyroiditis (HT), at first diagnosis of ATDs. The control group was composed of 100 class-matched individuals. Thyroid hormones and autoantibodies for ATDs diagnosis were measured by chemiluminescent immunoassay, using Liaison instrument (Diasorin, Italy). To evaluate HP infection, stool samples were tested by amplified enzyme immunoassay (Amplified IDEIA *H. pylori* STAR, Oxoid, United Kingdom). To detect Cag-A antibodies, serum samples were tested by enzyme-linked immunoassay method (ELISA, Radim, Pomezia, Italy). Results were analyzed by two-sided Fisher's exact test and the respective odds ratio (OR) calculated.  $P \leq 0.05$  was considered significant. **Results:** The presence of HP in stool samples was higher in GD group (43/52, 82%) than in HT group (28/60, 46%) and control group (43/100, 43%). A statistically significant interaction was found between HP positivity and Graves' disease ( $P \leq 0.0001$  vs control, OR 6.3), but not Hashimoto's thyroiditis. **Conclusions:** A possible role of HP infection in GD could be dependent on the different expression of adhesion molecules in the gastric mucosa, but further studies needs to display such hypothesis.

**IMMUNITY AND INFLAMMATION – INFLAMMATION**

**IMIN1. Study of the Modulation of Cytokine Release by Natural Compounds with Pharmacological Properties Using Cell-Based Systems**

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**Background:** The screening of the pharmacological properties of natural compounds (i.e., anti-inflammatory effects) may take advantage of some specific cell-based systems. Parthenolide (PTN) and Copaifeira langsdorffii (Copaiba) are natural compounds used to prevent and treat headache and migraine and in inflammatory diseases involving respiratory airways, genital-urinary apparatus and skin, respectively, but their effects at the cellular level are poorly understood. **Methods:** Mouse BV-2 microglia and human THP-1 monocyte cell lines were used. The nuclear translocation of nuclear factor (NF)- $\kappa$ B was evaluated by Western blotting analysis. The secretion of inflammatory cytokines (interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$  (TNF $\alpha$ )) was evaluated by immunometric assays (ELISA). **Results:** Treatment of BV-2 cells with 1  $\mu$ M PTN and of THP-1 cells with 10  $\mu$ M Copaiba oleoresin (OR), containing diterpene acids, diterpenes and sesquiterpenes, strongly reduced the NF- $\kappa$ B translocation to the cell nucleus induced by 1  $\mu$ g/mL lipopolysaccharide (LPS). In BV-2 cells, PTN reduced IL-6 secretion in a dose-dependent manner (-29% at 200 nM,  $P < 0.001$ ; -45% at 1  $\mu$ M,  $P < 0.001$ ; -98% at 5  $\mu$ M,  $P < 0.001$ ; ANOVA). Moreover, at 5  $\mu$ M (highest concentration tested) PTN also reduced TNF- $\alpha$  secretion (-54%,  $P < 0.001$ ). Preincubation of LPS-stimulated THP-1 monocytes with OR (dose-range: 0.1-10 mM), reduced the release of all tested cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ). **Conclusions:** The results obtained provide strong evidence that both cell-based models are useful to validate the anti-inflammatory properties of PTN and OR at the cellular level and suggest that they are related to inhibition of cytokine secretion and NF- $\kappa$ B nuclear translocation.

**IMIN2. Origins and Significance of Elevated Gamma-glutamyltransferase in Cystic Fibrosis Sputum**

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**Background:** Cystic fibrosis (CF) is an autosomal recessive disorder characterized by a chronic neutrophilic airways inflammation, increasing levels of oxidative stress and reduced levels of antioxidants such as glutathione (GSH). Gamma-glutamyltransferase (GGT), an enzyme induced by oxidative stress and involved in the catabolism of GSH and its derivatives, is increased in the airways of CF patients with inflammation, but the possible implications of its increase have not yet been investigated in detail. **Methods:** Sputum samples from 7 CF patients were analyzed by cytochemistry, HPLC-gel filtration, western blot and enzymology techniques. **Results:** GGT activity was found both in neutrophils and in ELF fluid, the latter significantly correlating with myeloperoxidase expression. In neutrophils, GGT was associated with intracellular granules. In the fluid, gel-filtration chromatography showed the presence of two distinct GGT fractions, the first corresponding to the human plasma b-GGT fraction, the other to the free enzyme. The same fractions were also observed in the supernatant of ionomycin and fMLP-activated neutrophils. Western blot analysis confirmed the presence of a single band of GGT immunoreactive peptide in the CF sputum samples and in isolated neutrophils.

**Conclusions:** In conclusion, our data indicate that neutrophils are able to transport and release GGT, thus increasing GGT activity in CF sputum. The prompt release of GGT may have consequences on all GGT substrates, including major inflammatory mediators such as S-nitrosoglutathione and leukotrienes, and could participate in early modulation of inflammatory response.

**IMIN3. Multi-step Regulation of Toll-Like-Receptor 4 Signalling by IL-10-dependent Anti-inflammatory microRNAs**

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**Background:** Toll-like receptors (TLRs) play a key role in detecting pathogens and initiating inflammatory responses that subsequently prime specific adaptive responses. To avoid excessive inflammation and consequent immunopathology, TLR signalling must be tightly regulated. Though microRNAs (miR) have emerged as important regulators in several biological processes, their functional role in the control of inflammatory responses remains incompletely understood. **Methods:** Bioinformatic analysis was performed using MiRanda software, luciferase assays were performed on target 3'-UTRs cloned in psi-Check2, THP-1 transduction was achieved using pRRL-based lentiviral constructs. **Results:** Stimulation of human monocyte/macrophages with LPS induced the expression of miR-187, miR-146b, and the cluster 99b/125a-5p/let7e at late time-points. Blocking experiments indicated that these miRs are all induced as part of the IL-10-dependent feedback loop, and all substantially decreased under chronicization conditions, mimicked by IFN $\gamma$  exposure. Bioinformatic analysis predicted and luciferase assays confirmed that receptors (TLR4, CD14), signal transducers (MyD88, IRAK1), transcription factors (IK-Bzeta), and effector molecules (TNF, IL-6, chemokines) involved in the TLR4 pathway are direct targets of these IL-10-dependent miRs. The biological relevance of this finding was confirmed by the significant reduction of LPS-dependent cytokine production achieved by overexpression of individual miRs in THP-1 cells.

**Conclusions:** We have identified a set of anti-inflammatory miRs, activated by IL-10 in human monocytes and macrophages, which are able to modulate TLR signalling acting at multiple steps of the signalling cascade, by direct targeting of receptors, adaptor/signalling proteins, and effector molecules. These miR candidate as new modulators of the response to LPS and are potentially involved in the resolution of inflammation.

**IMIN4. Recombination Is a Major Source of Genetic and Pathogenic Diversification of Group A Streptococcus Serotype M89 Strains**

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**Background:** Group A *Streptococcus pyogenes* (GAS) is a genetically diverse human pathogen having > 150 serotypes. Serotype M89 strains are a common cause of GAS pharyngeal and invasive infections. Recent studies indicate M89 strains may be increasing in prevalence. Over the last 15 years in Italy, two large epidemic outbreaks were caused by macrolide resistant M89 strains. M89 epidemic strains differed in gene content encoding virulence factors and macrolide resistance. We used comparative genomics to assess serotype M89 genetic diversity and its contribution to GAS pathogenesis. **Methods:** Next-generation DNA sequencing was

conducted for 22 isolates (Italy = 5, Canada = 1, New Zealand = 1, US = 15). Complete genomes were determined for 1 Italian and 1 US strain. Polymorphisms were identified genome-wide for the cohort and used to infer genetic relationships. **Results:** The genome of Italian strain 11610 is 1,709,407 bp and lacks prophages. Italian epidemic isolates differed pairwise by ~32 core-genome SNPs. The genome of US strain MGAS11027 is 1,786,881 bp and has 2 prophages encoding secreted virulence factors. US M89 strains differed pairwise by ~397 SNPs. Italian M89s differed from US M89s by ~2064 SNPs. SNPs among US M89s, and differing US from Italian M89s, were nonrandomly clustered. **Conclusions:** SNPs among the M89 strains exceeded that among other GAS serotypes. Most SNPs were attributed to discrete recombination events involving genes encoding virulence factors including surface adhesins, antimicrobial peptides, and secreted anti-immune response proteins. These findings demonstrate that genetic recombination strongly contributes to diversification of M89 pathogenic capacity.

**IMIN5. Platelets Amplify IL-6 Trans-signaling**

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**Background:** Human platelets are a key element linking hemostasis, inflammation, and tissue repair. Produced by a broad spectrum of cell types in the cardiovascular system, IL-6 has become a marker of vascular inflammation, associated with a variety of clinically significant outcomes. Recent literature showed a trans-signaling molecular mechanism by which IL-6 could affect leukocyte-recruitment through the release of soluble IL-6 receptor (sIL-6R). We investigated the potential role of platelets in this mechanism. **Methods:** For each experiment human platelets were isolated from healthy donor single buffy-coat, free of leukocytes. Short time stimulations were performed with thrombin, IL-6, the soluble specific IL-6Rs and Hyper-IL6, alone or in combination; Hyper-IL6 is a fusion protein of human IL-6 covalently linked to the human sIL-6R and is 100- to 1000-fold more active than the natural complex, usually used for *in vitro* experiments. Western blotting, FACS and ELISA were used to analyze platelet reactivity. **Results:** a) activated platelets can release biologically active sIL-6R, b) the complex IL-6/sIL-6R increases platelet IL-6R and gp130 expression, and c) the complex IL-6/sIL-6R induces STAT3 phosphorylation. **Conclusions:** We point out the potential role of platelet/IL-6 trans-signaling interaction, which could be another not irrelevant link between inflammation and hemostasis.

**IMIN6. Inhibition of Cytokine-Induced Signaling Pathways and Target Gene Expression by Plant Components in Pancreatic Beta Cells**

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**Background:** We have previously found that the extract of *Hypericum perforatum* (St John's wort), SJW and its component hyperforin (HPF) protect pancreatic beta cells against the cytotoxic effects of cytokines, by acting as powerful inhibitors of the transcription factors STAT-1 and NF- $\kappa$ B. Aims of this study were: a) to explore the time course of STAT-1 inhibition in the INS-1E cell line; b) to further clarify the mechanisms of the regulatory activity of SJW and HPF on the cytokine signaling pathways and expression of target genes. **Methods:** INS-1E cells, exposed to mixtures of IFN- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$  with/without SJW or HPF, were used for RT-qPCR gene expression analysis and assessment of phosphorylated components of STAT-1, NF- $\kappa$ B and MAPK pathways by western blotting. **Results:** SJW and HPF down-regulated STAT-1 even after their removal from the incubation medium before the addition of cytokines or when added 15-30 min following cytokines. The vegetal compounds dose-dependently prevented cytokine-induced STAT-1 phosphorylation in both tyrosine and serine residues. NF- $\kappa$ B activation was hindered through suppression of the p65 subunit phosphorylation and interference with the inhibitory subunit I $\kappa$ B. MAPK cascade was also modulated by SJW and HPF through dose-dependent restriction of ERK1/2 and p38 MAPK phosphorylations. SJW and HPF restrained cytokine-induced mRNA expression of pro-inflammatory genes (e.g., iNOS, CXCL9, CXCL10, ICAM-1) and partially corrected the cytokine-induced imbalance between anti- and pro-apoptotic factors. **Conclusions:** SJW and HPF, by counteracting crucial mechanisms of cytokine-induced inflammatory and apoptotic alterations, represent interesting pharmacological tools for prevention or limitation of dysfunction and beta-cell loss in diabetes.

**KIDNEY DISEASES**

**KD1. Study of Molecular and Functional Effects Induced in Tubular Primary Cell Cultures by High Glucose Treatment**

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**Background:** Tubulointerstitial fibrosis is an important component of renal injury in diabetic nephropathy. Tubular and tubulointerstitial cells may contribute in different ways to the fibrotic phenotype. We analyzed the effects of high glucose treatment on epithelial tubular cells *in vitro* to evaluate their contribution to development of renal fibrosis. **Methods:** Control (DMEM 100mg/dl D-glucose) and treated (DMEM 450mg/dl D-glucose) tubular primary cell cultures have been analysed for gene and miRNA expression by Real Time PCR, FACS, Western blot, Immunofluorescence, and functionally characterized by adhesion and migration assays. Conditioned media from control and treated cells were submitted to Multiplex Cytokine analysis and evaluated for their ability to activate NIH3T3 fibroblasts. **Results:** Treated tubular cells show a few phenotypic features of EMT, an increase of proliferation and a decrease of migration properties. In these treated cells actin filaments are reorganized in stress fibers with an increase of focal adhesions. The expression of nonreceptor tyrosine kinase Arg, known to regulate cellular morphology and adhesion through RhoGTPases, is decreased and show a different isoform pattern in treated cultures respect to control. Finally, cytokine analysis of culture media evidences an increase of MCP-1, GM-CSF and TGF- $\beta$ 1 level in treated cells. Conditioned media from treated cells are able to increase fibroblast proliferation. **Conclusions:** Although high glucose treatment induced cytoskeletal changes and the secretion of pro-inflammatory and fibrotic cytokines in tubular primary cell cultures, it was unable to induce a complete EMT phenotype.

**KD2. Exposure to Low- Versus Iso-Osmolar Contrast Agents Induces NADPH-dependent Reactive Oxygen Species (ROS) Generation in a Cellular Model of Renal Injury**

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**Background:** Contrast-induced nephropathy (CIN) represents the third cause of hospital-acquired acute-renal-failure. In this study we aimed to determine whether the use of iso-osmolar iodixanol is less nephrotoxic than low-osmolar iohexol and iopamidol in a cellular model. **Methods:** X-ray attenuation of iohexol, iopamidol and iodixanol was assessed at equimolar iodine concentrations. Human renal proximal tubular cells were incubated with equally attenuating solutions of CM. Cytotoxicity was assessed by trypan-blue-testing, MTT-assay and AnnV/PI-assay to detect apoptosis and necrosis. ROS-production was assessed by DCF-assay and NADPH-oxidase-activity was checked by chemiluminescence method. **Results:** Yielding the same x-ray attenuation, CM-cytotoxicity was assessed at equimolar iodine-concentrations. At trypan-blue-testing, there were more necrotic cells after incubation with 50, 100, 150 and 270mg/ml of iohexol and iopamidol than after incubation with equal concentrations of iodixanol ( $P < 0.005$ ). Iohexol and iodixanol at 20 and 50mg/ml induced comparable inhibition of MTT-conversion with a dose-dependent-effect, whereas iopamidol showed a marked cytotoxic effect, as compared with iodixanol (26.0 vs. 54.9% at 20mg/ml and 23.2 vs. 36.8% at 50mg/ml of undamaged control cells for iohexol and iodixanol, respectively;  $P < 0.05$ ). Moreover, both iohexol and iopamidol induced more necrosis and apoptosis than iodixanol with a dose-dependent effect (iodixanol vs. iopamidol: for necrosis 1.9 vs. 1.1% at 20mg/ml and 2.8 vs. 0.9% at 50mg/ml,  $P < 0.05$ ; for apoptosis 45.6 vs. 36.9% at 20mg/ml and 52.0 vs. 40.0% at 50mg/ml,  $P < 0.05$ ). ROS-generation was higher for iopamidol and iohexol as compared with iodixanol (after 40min 21263  $\pm$  2124 and 8985  $\pm$  1601 vs. 4430  $\pm$  801 AU at 20mg/ml,  $P < 0.05$ ; 9844  $\pm$  1124 and 8113  $\pm$  101 vs. 4076  $\pm$  601 AU at 50mg/ml,  $P < 0.05$ ). NADPH-oxidase-activity significantly increased after exposure to iopamidol and iohexol with a dose-dependent effect, as compared with iodixanol (27.6 and 50.5 vs. 8.9% over the basal at 20mg/dL,  $P < 0.05$ ; 50.5 and 71.1 vs. 35.8% over the basal at 50mg/dL,  $P < 0.05$ ). **Conclusions:** At angiographic concentrations, iodixanol induces fewer cytotoxic effects on cultured tubular cells than iohexol and iopamidol by inducing lower amount NADPH-dependent ROS-generation.

**NEOPLASIA – ADVANCES IN MOLECULAR CANCER THERAPIES**

**NAMT1. Photochemical and Photobiological Evaluation of Fluoroquinolones as Anticancer Agents**

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**Background:** Fluoroquinolones are antimicrobial agents used against many infectious diseases. When UVA-irradiated, some of them exert phototoxic and mutagenic effects and those showing the strongest photogenotoxicity have a second fluorine atom in position 8 (other than the one in position 6). The mechanism of action proposed is based on ROS formation, but studies in solution demonstrated a new photochemical reaction producing a triplet-aryl cation able to attack DNA bases. An oxygen-independent photodynamic agent would be a good strategy against cancer, because of its low oxygen pressure. In this study, the photobiological activity of commercially available or newly synthesized fluoroquinolones, optimized to act via aryl cation, have been investigated. **Methods:** Ciprofloxacin, lomefloxacin and ofloxacin photoactivity were evaluated in A431 and HeLa cancer cells in terms of intracellular localization, phototoxicity, DNA damage, cell growth inhibition and apoptosis induction. Cells were incubated with each compound for 24h, then UVA-irradiated both at normal oxygen partial pressure and under hypoxic conditions. **Results:** Fluoroquinolones appeared as small blue spots within the cytoplasm. Most of them colocalized with lysosomes and less with mitochondria. Among the three fluoroquinolones, ciprofloxacin showed the highest photocytotoxicity, whereas both ciprofloxacin and lomefloxacin markedly impaired cell cycle progression inducing apoptotic cell death. Ciprofloxacin showed a higher damaging potential on DNA plasmid, followed by lomefloxacin, then by ofloxacin. Considerable amount of DNA damage, both as SSBs and oxidized pyrimidine or purine bases, was detected in lomefloxacin- or ciprofloxacin-treated cells. **Conclusions:** Fluoroquinolones' photobiological effect was influenced by the oxygen pressure. A bi-phasic mechanism of action can be envisaged.

**NAMT2. A Novel Molecular Pathway for Caveolin-1 as an Oncopromoter in Glioblastoma Cells**

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**Background:** Caveolin-1 is an essential structural constituent of caveolae implicated in mitogenic signalling, oncogenesis, angiogenesis, neurodegenerative diseases and senescence. Its role as a tumor suppressor gene or as a tumor promoter seems to strictly depend on cell type and tumor stage/grade. The high expression of caveolin-1 in some tumors *in vivo* is associated with increased tumor aggressiveness, metastatic potential and suppression of apoptosis. **Methods:** In glioblastoma A172, CRS-A2, LI cell lines we found Cav-1 expressed at high levels. Silencing of Cav-1 gene was performed using small interfering RNA (siRNA). Cells were grown to 30-40% confluence and transfected for 72 h with 40 nM siRNA using Oligofectamine Reagent. Non-specific siRNA was used as a negative control. Cav-1 expression was assayed by immunoblot analysis. Cell proliferation was tested by MTT assay. **Results:** Results in the A172 cell line are presented here and show that siRNA-mediated down-regulation of Cav-1 caused stable arrest of proliferation. A marked reduction of cyclin D1 and of CDK4 expression was evident in the cells transfected with Cav-1 siRNA and consequently of phosphoRb. Furthermore, a significant decrease of the expression of Src and p38 $\alpha$  and of their down-stream effector STAT3 was evident. **Conclusions:** Together, these findings indicate that Cav-1 silencing induces an arrest of human glioblastoma cells proliferation by a new inhibitory pathway in this tumor, the most aggressive of the primary central nervous system tumors, and provide new insights into the molecular mechanisms underlying the pro-survival and tumor-promoting functions of Cav-1.

**NAMT3. Synergy Between HDACi and PARP Inhibitor on Breast Cancer Cells**

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**Background:** An important strategy for cancer treatment consists of the contemporary use of different compounds. In this research we have investigated the combination of suberoylanilide hydroxamic acid (SAHA), a histone deacetylases inhibitor (HDACi), and a PARP Inhibitor (PJ34) on proliferation and gene expression of breast cancer cell lines. HDAC inhibitors and PARP Inhibitors have shown antitumor activity across a broad variety of hematologic and solid tumors in both preclinical studies and clinical trials. **Methods:** MDA 468, MDA 231, MDA 157 and T47D breast cancer cell lines, were investigated. In a first set of experiments we tested whether various doses of SAHA and PJ34 have synergic effect on cell

proliferation and apoptosis. Then the synergic dose has been used to treat the cell lines to quantify the mRNA of the NIS gene, with real time PCR. **Results:** On MDA 157 we observed a strong synergy on cell viability between the two compounds. On this cell line synergy between SAHA and PJ34 is observed also at levels of apoptosis. The effect of SAHA and PJ34 was investigated also on the expression of Sodium Iodide Symporter (NIS) in MDA 157 and MDA 468 cell line. On MDA 468 cell line synergy among the two compounds, on NIS gene expression, was very strong. **Conclusions:** These results suggest that combinations between HDACi and PARP inhibitors may be proposed in breast cancer treatment.

**NAMT4. PDGFR $\alpha$  Signaling in Liver Regeneration Reveals Novel Redundancies: Implications in Hepatocellular Cancer**

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**Background:** Platelet derived growth factor receptor- $\alpha$  (PDGFR $\alpha$ ) is known for its role in mesenchymal cells such as fibroblasts and endothelial cells and other cells like neurons. We have recently identified low expression of PDGFR $\alpha$  in adult hepatocytes, which is up-regulated in hepatocellular cancer. **Methods:** To determine the role and regulation of PDGFR $\alpha$  in hepatocyte biology, we generated hepatocyte-specific PDGFR $\alpha$  knockout mice (KO). We examined liver regeneration (LR) in KO and control (WT) mice after partial-hepatectomy (PH). **Results:** Loss of PDGFR $\alpha$  in hepatocytes was evident at 2 months, albeit no gross, histological or biochemical anomaly was discernible. We identified increased total and active PDGFR $\alpha$  protein at 24hr post PH. Loss of PDGFR $\alpha$  in hepatocytes did not engender any changes in hepatocyte viability (TUNEL) at any timepoint during LR; however, we observed increased hepatocyte proliferation (PCNA) at 72hr during LR after PH. Interestingly, we observed enhanced expression of both EGFR and MET in the KO livers at various timepoints during LR. In addition, *in vitro* analyses using primary mouse hepatocytes shows that activation of hepatocyte PDGFR $\alpha$  abrogates, whereas its blockade enhances, EGF/HGF induced hepatocyte proliferation. **Conclusions:** Thus, although PDGFR $\alpha$  increase is conspicuous during LR, its loss in hepatocytes appears to be possibly compensated for by both EGFR and MET, which may explain the lack of overt phenotype in KO mice during LR. Also, targeting PDGFR $\alpha$  in HCC cells may lead to EGFR/Met activation necessitating sequential inhibition of these pathways for improved therapies. (CATER fellowship (NIH T32 EB001026-05) to PA; 1R01DK62277 and 1R01CA124414)

**NAMT5. Impact of Histone Deacetylase Inhibitors SAHA and MS-275 on IL-8 Synthesis in Human Melanoma Cells**

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**Background:** Elevated interleukin-8 (IL-8) levels have been observed in patients with metastatic melanoma, and histone deacetylases inhibitors (HDACis) have been shown to influence melanoma progression. In most instances, HDAC inhibitors were positively acting in cooperation with inducers of IL-8, whereas in other cases IL-8 expression was down-regulated by HDACis. This study aims at investigating whether the HDACis SAHA and MS-275 affect IL-8 expression in melanoma cells. **Methods:** Cutaneous and uveal melanoma cell lines were treated with HDACis and IL-8 mRNA and protein were determined by real time-PCR and enzyme-linked immunosorbent assay. The protein content of the main transcription factors involved in IL-8 gene regulation and their binding to IL-8 promoter were evaluated by western blot and chromatin immunoprecipitation assay. Cell proliferation and apoptosis rates were also investigated. **Results:** HDACis strengthened IL-8 mRNA and protein expression. In parallel, increased cell proliferation and reduced rate of apoptosis were observed. The observed activation of IL-8 by HDACis correlated with increased protein levels of c-Jun. On the contrary, CHOP, Rel-A and C/EBP $\beta$  synthesis was not affected. Interestingly, SAHA and MS-275 induced c-Jun binding to the IL-8 promoter as well as c-Jun transcription by favoring the recruitment of the preinitiation complex (RNA polymerase II and TFIIB) to the c-Jun promoter. **Conclusions:** Data reported here indicate that the inhibition of class I HDAC activity is a requisite to activate IL-8 expression in cutaneous as well as uveal melanoma. The increase of IL-8 was mediated by c-Jun promoter activation and was accompanied by enhanced cell proliferation and reduced apoptosis.

**NAMT6. Development of a Leptin Antagonist Peptide: Implications for Breast Cancer**

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**Background:** The role of the obesity cytokine leptin in breast cancer progression has raised interest in interfering with leptin's actions as a valuable therapeutic strategy. Leptin interacts with its receptor through three different binding sites: I-III. Site III is crucial for the formation of an active leptin-leptin receptor complex and in its subsequent activation. Amino acids 39-42 (LDFI) were shown to contribute to leptin site III and their mutations in alanine resulted in muteins acting as typical antagonists. Based on this design strategy, we synthesized the unmodified leptin fragment LDFI and evaluated its activity in both estrogen receptor-positive and -negative breast cancer cells. **Methods:** The peptide was synthesized by CEM-Liberty microwave-assisted automated-synthesizer, and characterized by 1H-NMR spectroscopy. We assessed signaling pathway activation by immunoblotting analysis, proliferation by anchorage-dependent and -independent growth assays and migration by wound-healing assays. **Results:** The LDFI-peptide abolished the leptin-induced phosphorylation of its downstream effectors, as JAK2/STAT3/Akt/MAPK, without any agonistic activity. These results correlated with a reduction in anchorage-dependent and -independent growth as well as migration of breast cancer cells. Importantly, the LDFI fragment reversed the leptin-mediated up-regulation of its gene expression, as an additional mechanism able to enhance the peptide antagonistic activity. The described effects were specific for leptin signaling, since the developed peptide was not able to antagonize the other growth factors' actions on signaling activation, proliferation and migration. **Conclusions:** We demonstrate that the unmodified LDFI-peptide acts as a full leptin antagonist and could become an attractive option for breast cancer treatment, especially in obese women.

**NAMT7.  $\beta$ -Catenin-Mutated Human Hepatocellular Carcinoma (HCC) Cells Show Features of Glutamine Addiction**

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**Background:** High consumption of anaplerotic substrates is a hallmark of cancer cells, which must couple energy production with macromolecular synthesis to sustain a rapid growth. Although most tumors use glucose for anaplerosis, some cancer cells rely on glutamine and, hence, are defined glutamine-addicted. **Methods:** HepG2 and Huh-7 were grown in low-glucose DMEM supplemented with 10% FBS, 4mM Gln and antibiotics. Glutamine depletion was obtained with L-asparaginase (1U/ml) w/o the GS inhibitor MSO (1mM). Glutamine Synthetase (GS) and SNAT2 mRNA levels were measured with qRT-PCR. Amino acid content was assessed with HPLC. mTOR activation was evaluated determining downstream target phosphorylation with Western Blot. **Results:** We found that  $\beta$ -catenin-mutated human hepatocellular carcinoma (HCC) HepG2 cells express high mRNA levels for GS and the Gln transporter SNAT2, even when exposed to supra-physiological Gln, and maintain a larger intracellular Gln pool than  $\beta$ -catenin w/t Huh-7 counterparts. However, paradoxically, HepG2 cells also exhibit enhanced sensitivity to Gln starvation and increased accumulation of Gln-mimetic GS inhibitors, leading to inappropriate signalling through amino acid dependent pathways, such as mTOR. Consistently, GS inhibitors synergise the cytotoxic effect of the glutaminolytic enzyme L-asparaginase. Preliminary evidence *in vivo* shows that the combined treatment of nude mice with L-asparaginase and the GS inhibitor MSO was well tolerated and lowered Gln content in serum, liver and xenografted HepG2 tumors, leading to impaired tumor growth. **Conclusions:** Thus, the glutamine addicted phenotype, exhibited by  $\beta$ -catenin-mutated HCC cells *in vitro*, may indicate tumor sensitivity to glutaminolytic treatments *in vivo*.

**NAMT8. Farnesoid X Receptor Activation Induces Apoptosis and Inhibits Leydig Xenograft Tumor Growth**

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**Background:** Leydig cell tumors are the most common tumors of the gonadal stroma and represent about 3% of all testicular neoplasms. In most cases, Leydig cell tumors are benign; however, if the tumor is malignant, no effective treatments are currently available. We have recently reported that farnesoid X receptor (FXR) is expressed in R2C Leydig tumor cells, and it reduces the estrogen-dependent R2C cell proliferation by negatively regulating aromatase expression. **Methods:** R2C tumor xenograft models for *in vivo* studies. Immunoblotting, DNA laddering and Tunnel assays for apoptosis. Immunoblotting, RT-PCR, reporter-gene assays, mutagenesis experiments, EMSA and ChIP analysis to investigate the molecular

mechanisms. **Results:** We demonstrated that treatment with GW4064, a specific FXR agonist, markedly reduced tumor growth in R2C xenograft models and induced apoptosis in R2C Leydig cells both *in vitro* and *in vivo*. Indeed, FXR ligands induced an enhanced PARP cleavage, a marked DNA fragmentation and a strong increase in the number of apoptotic nuclei. Moreover, FXR activation up-regulated p53 mRNA and protein levels along with an increased expression of its downstream effector p21WAF1/Cip1. Functional experiments showed that FXR ligands up-regulated p53 promoter activity. This occurs through an increased binding of FXR/NF- $\kappa$ B complex to the NF- $\kappa$ B site located within p53 promoter region. **Conclusions:** These data demonstrate that the induction of apoptotic pathways may represent an additional mechanism through which FXR ligands inhibit Leydig tumor cell growth. From a therapeutic standpoint, strategies aimed to activate FXR might be useful for the treatment of Leydig cell tumors.

**NAMT9. Omega-3 Ethanolamides Induce Autophagy through PPAR-gamma Activation in Breast Cancer Cells**

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**Background:** The omega-3 long chain polyunsaturated fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), elicit antiproliferative effects in cancer cell lines and in animal models. Dietary DHA and EPA can be converted to their ethanolamide derivatives, DHEA and EPEA, respectively; however, few studies are reported on their anticancer activities. **Methods:** Proliferation of MCF-7 and MCF-10 breast cells using MTT-assays. Apoptosis and autophagy by DNA fragmentation and monodansylcadaverine labelling, respectively. Transfection experiments using peroxisome proliferator-activated receptor (PPAR)-response-element-reporter plasmid. mRNA and protein levels by RT-PCR, immunoblotting and immunofluorescence analyses. **Results:** We demonstrated that DHEA and EPEA reduced cell viability in MCF-7 breast cancer cells whereas they did not elicit any effects in MCF-10A non-tumorigenic breast epithelial cells. Since, DHA and EPA are both ligands of PPAR-gamma, we sought to determine whether PPAR-gamma may mediate DHEA and EPEA actions. In MCF-7 cells, both compounds enhanced PPAR-gamma expression, stimulated the transcriptional activity of a PPAR-response-element-reporter plasmid and increased the expression of the oncosuppressor PTEN, a well known PPAR-gamma target gene. PTEN up-regulation caused the inhibition of Akt-mTOR pathways which in turn leads to the activation of either apoptotic or autophagic processes. DHEA and EPEA induced phosphorylation of Bcl-2 promoting its dissociation from beclin-1 which resulted in autophagy induction. We also observed an increase of beclin-1 and LC3 expression. These effects appeared to be PPAR-gamma dependent. **Conclusions:** DHEA and EPEA acting as PPAR-gamma ligands exert antiproliferative effects by inducing autophagy in breast cancer cells highlighting their potential use in adjuvant breast cancer therapies.

**NAMT10. AntiTumor Activity of Epratuzumab/Saporin-S6**

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**Background:** CD22 represents an attractive molecular target for B-cell neoplasm therapy with immunoconjugates. Epratuzumab, a humanized anti-CD22 mAb has induced tumor regression in preclinical and phase I/II clinical evaluations in patients with indolent or aggressive lymphoma. The results obtained in clinical studies encourage the attempts to improve the Epratuzumab effectiveness, i.e. by conjugation to toxic molecules (immunotoxins). Among plant toxins, the most frequently employed to generate immunotoxins are ribosome-inactivating proteins (RIPs). Saporin-S6 is a RIP often used to construct immunotoxins tested in clinical trials. **Methods:** The humanized anti-CD22 mAb epratuzumab was conjugated to the toxic enzyme saporin-S6, a type I ribosome-inactivating protein (RIP). The antitumor effect of this immunotoxin has been studied *in vitro* on CD22+ cell lines and *in vivo* in an NHL/SCID mice model. **Results:** The epratuzumab/saporin-S6 immunotoxin was specifically toxic to five different CD22+ lymphoma cell lines while sparing non-target CD22- cells. The cytotoxic effect was demonstrated *in vitro* by the complete inhibition of protein synthesis, strong induction of caspase activity, complete loss of viability and total suppression of clonogenic growth of CD22+ cell lines. The immunotoxin showed potent antitumor activity in a SCID mouse Raji xenograft model of human aggressive lymphoma. **Conclusions:** Our results indicate that it is possible to augment epratuzumab toxicity in target cells by linking the antibody to saporin-S6. An excellent therapeutic index was achieved by this immunotoxin in animals. These results may encourage further evaluation of this conjugate in a phase I clinical trial.

**NAMT11. Cell Therapy of Human Cancer: uPAR truncation by Engineered Endothelial Progenitor Cells (EPC) as Intra-Tumoral Shuttles of Anti-Invasion/Anti-Metastatic MMP12**

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**Background:** The onset of angiogenesis ("angiogenic switch") is a critical step in tumor development and plays a relevant role in cancer progression. The term "angiogenesis" connotes the development of new vessels from pre-existing ones. Identification of bone marrow-derived endothelial progenitor cells (EPCs) in peripheral blood has highlighted an alternative mechanism of angiogenesis ("postnatal vasculogenesis"). However only a subset of EPC, termed endothelial colony-forming cells (ECFCs), have been shown to display the characteristic of a true endothelial cell (EC) progenitor and to possess the ability to form de novo blood vessels *in vivo*. Many studies suggest an important role of EPC in tumor vascularization and metastasis. ECFCs have been shown to home within tumors and therefore can be used as cellular vehicle to deliver anticancer agents. The membrane-associated plasminogen activation system (urokinase-type plasminogen activator, uPA; uPA receptor, uPAR) is critical in angiogenesis as well as in invasive properties of cancer cells. Only full length uPAR fosters invasion and angiogenesis. Truncation of uPAR domain 1 by matrix metalloproteinase-12 (MMP12) impairs cell invasion and angiogenesis. **Methods:** Design and construct of lentivirus encoding MMP12 and delivery of the vector into ECFC. **Results:** Our preliminary data show that the "gain of function" of MMP12 activity in ECFC shuttles can control tumor progression and angiogenesis on several melanoma cell lines **Conclusions:** *Ex vivo* manipulated ECFCs overexpressing MMP12 could be used as cellular vehicle to deliver MMP12 anticancer agent, providing a new way to block growth and metastasis of those tumor which heavily depend on uPAR to perform invasion.

**NAMT12. Inhibition of p38MAPK: A Combined Strategy for Sensitizing Neuroblastoma Cells to Etoposide Through the Modulation of Pro-Inflammation Markers**

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**Background:** The tumor-associated inflammatory response has the paradoxical effect of enhancing tumorigenesis and tumor progression. In this regard, there is increasing evidence that the interaction of several pro-inflammatory chemokine receptors with corresponding chemokine ligands are implicated in the growth and invasive phenotype of neuroblastoma (NB). Moreover, it has been reported that in NB cells COX-2 increases migration and modulates the expression of the ICAM-1, an inducible surface glycoprotein that mediates adhesion-dependent cell-to-cell interactions. **Methods:** The present study has been performed on HTLA-230, a MYCN-amplified NB cell line exposed to etoposide alone or in association with the drugs targeting the intracellular signaling pathways. The oxidative and pro-inflammatory markers have been evaluated by fluorescence microscopy analysis and molecular biology techniques. **Results:** We provide the evidence that HTLA-230 are highly resistant to etoposide which induces a dose-dependent ROS overproduction, DNA double-strand breaks and p38MAPK activation. Therefore, the treatment with etoposide combined with SB203580, an inhibitor of p38MAPK activity has been found to decrease cell viability and tumorigenicity, counteract stem cell development and slow down the cell migration and invasion. In this context, the expression of COX-2, ICAM-1 and CXCR4 is down regulated, the formation of capillary-like structures is prevented, by generating a phenotype inadequate for tumor development. **Conclusions:** Collectively, our results suggest that clinical trials of p38MAPK inhibitors, in combination with standard chemotherapy, acting on the modulation of pro-inflammation markers, could be a novel strategy to counteract NB resistance and relapse. (Grants from PRIN 2008N9N9KL\_002, PRIN 2009M8FKBB\_002 and Genoa University).

**NAMT13. Epigallocatechin Gallate as a New Effective Estrogen Receptor Alpha (ER- $\alpha$ ) Down-Regulator in Breast Cancer**

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**Background:** Increasing exposure of the breast to estrogens and other sex hormones is an important cancer risk factor and since estrogens are mitogenic to breast epithelial cells, the magnitude of their effects may be determined by the levels of estrogen receptors (ERs) expressed in the breast. The strong correlation between ER- $\alpha$  expression, breast disease pathophysiology and therapeutic response, make

ER down-regulators of significant clinical interest. In recent years epigallocatechin gallate (EGCG), a polyphenolic compound found in green tea, evidenced chemopreventative and antitumor properties. **Methods:** Here we investigate whether EGCG effects on breast cancer cell proliferation could be detected following extended treatments with low doses of the catechin. We tested ER+ PR+ breast cancer cell lines, including T47D and MCF-7 cells. **Results:** We report that EGCG causes concomitant PR nuclearization and down-regulation of ER- $\alpha$  protein, mRNA and gene promoter activity. These events appear specifically PR-B dependent, since they are drastically abrogated with PR-B siRNA. EMSA and ChIP assay reveal that, upon EGCG treatment, PR-B is recruited at the half PRE site on ER- $\alpha$  promoter, together with a corepressor complex containing NCoR and HDAC1. RNA polymerase II is displaced, indicating that the chromatin in this region is in a less permissive environment for gene transcription. Finally we define the functional significance since EGCG produces a significant inhibition of ER+ PR+ breast cancer cell proliferation and anchorage independent growth. **Conclusions:** These results address how EGCG/PR-B signaling may be considered as useful tool to be exploited in the adjuvant settings for treatment of breast cancer.

**NAMT14. Generation of Mutants of *Helicobacter pylori* L-Asparaginase**

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**Background:** *Helicobacter pylori* L-asparaginase is a recently isolated bacterial factor able to inhibit the cell-cycle of exposed cells, but also a potential platform to develop new anti-cancer drugs, due to its remarkable selectivity for L-asparagine. **Methods:** To generate new, useful variants of the enzyme, site directed mutagenesis and random mutagenesis are being used to introduce modifications of the protein. The latter method required finding a powerful selection method to isolate interesting mutants. **Results:** Site-directed mutants generated for L-asparaginase show different levels of activity both towards L-asparagine and L-glutamine. Selection of random mutants is still ongoing. **Conclusions:** Dissection of L-asparaginase activity towards different substrates can be useful to generate better anti-cancer therapeutics.

**NAMT15. The Prolyl-Isomerase Pin1 Represents a Regulator of Notch3 Protein Functional Activity**

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**Background:** Pin1 is a prolyl-isomerase involved in the regulation of signal transduction. Its activity is crucial in the control of DNA damage checkpoint pathways and in the modulation of cell proliferation. Alterations of Pin1 are often associated with human malignancies. In particular, a recent report demonstrated the specific role of Pin1 in regulating Notch1 activity, finally contributing to the development of human breast cancer. Deregulated Notch3 signalling has been implicated in T cell leukemogenesis; however, the mechanisms underlying Notch regulation in leukemia remain incompletely clarified. Our purpose is to demonstrate that: a) Notch3 is a new target of Pin1 prolyl-isomerase; b) Pin1 may represent a novel target in T-ALL therapeutic approach. **Methods:** Transient co-transfection in HEK293T cells; immunoprecipitation assay; Pin1 silencing with lentivirus plasmids and Pin1 inhibitor (Juglone) treatment of established Notch3 transgenic murine cell line (N3-232T). **Results:** In this study we demonstrate that Notch3 protein is a new target of Pin1 isomerase and that their direct interaction depends on the presence of crucial phosphorylated sites (Ser/Thr-Pro) within Notch3 protein. Importantly, the inhibition of Pin1 function influences the intracellular localization of Notch3 protein in Notch3-IC overexpressing T lymphoma cell line, by sequestering Notch3 in cytoplasmic sub-organelles, finally markedly interfering with Notch3-dependent cell proliferation. **Conclusions:** We previously showed that the overexpression of Notch3 sustains T cell leukemogenesis and characterizes murine and human T-ALL. Together our present observations suggest a specific role of Pin1 in the regulation of Notch3 protein activity, offering new insight into the pathogenesis and therapeutic approach of Notch3-dependent T-cell leukemia.

**NAMT16. The Maintenance of Gefitinib Inhibits Migration and Epithelial-Mesenchymal Transition of Non-Small Cell Lung Cancer Cell Lines that Have Become Resistant after Prolonged Gefitinib Treatment**

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**Background:** It is well known that in patients harbouring EGFR activating mutations, disease progression occurs after a median of 10-14 months after the beginning of

TKIs therapy. The most frequent mechanisms of resistance are the acquisition of the T790M mutation and MET amplification. In standard oncology practice, progression during a specific therapy leads to initiation of a different treatment. Nevertheless, there are indications suggesting that a second EGFR-TKI treatment could be effective in patients who had benefited from the initial gefitinib therapy. This preclinical study has been designed to test the effects of maintaining gefitinib in NSCLC cell lines that have become resistant to gefitinib treatment. **Methods:** Experiments were performed on HCC827GR5, a cell line carrying activation mutation but with acquired resistance to gefitinib. Proliferation was determined by MTT, migration and invasion were determined by using Boyden chambers; signaling transduction proteins and epithelial-mesenchymal transition (EMT) markers were evaluated by Western blotting. **Results:** Gefitinib withdrawal did not modify resistance phenotype regarding the proliferation index and resistance to the drug. On the contrary, in the absence of gefitinib, resistant cells showed more migrating and invasion capability and the induction of typical EMT markers. The maintenance of gefitinib, instead, reduced cell migration, cell invasion and EMT. **Conclusions:** These results indicate that the maintenance of gefitinib might be important to control important malignant phenotypes of tumour cells such as loss of epithelial features and the acquisition of invasiveness.

**NAMT17. DAX-1 at the Crossroads Between Androgens and Aromatase: A Novel Mechanism in the Inhibition of Estrogen-Dependent Cancer Cell Proliferation**

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**Background:** Sex hormones, estrogens and androgens, are trivial to determine biological response in a tissue- and gender-specific manner. Estrogens, synthesized from androgens by aromatase, influence the pathological processes of several hormone-dependent cancers since they are thought to be the driving force for the formation of breast, endometrial, ovarian and Leydig cell tumors. Likely, the adequate androgen/estrogen ratio represents the most clinically relevant factor in this process; however the molecular mechanism underlying the androgen/estrogen essential balance still needs to be clarified. Here, we investigated the androgen-dependent modulation of DAX-1, a transcriptional corepressor of genes regulated by steroidogenic factor-1 (SF-1), such as aromatase, as well as of agonist-bound estrogen-receptor. **Methods:** Cell proliferation by MTT-assays. mRNA and protein levels by RT-PCR, immunoblotting and immunofluorescence analyses. Functional studies by luciferase/DAPA/EMSA/ChIP assays. **Results:** Using human breast cancer and rat Leydig tumor cells as experimental systems we demonstrated that ligand-activated androgen receptor (AR) induces the expression of DAX-1 by enhancing its promoter activity. These effects are mediated by direct binding of AR to a newly identified androgen response element within the DAX-1 proximal promoter. In turn, increased DAX-1 inhibits SF-1-mediated aromatase expression, thus reducing in situ estrogen production which is responsible for the estrogen-dependent proliferation of carcinoma cells. **Conclusions:** DAX-1 appears to be a specific androgen-target gene in breast and Leydig tumor cells. Since DAX-1 expression has been shown to influence cell growth by reversing estrogen-dependent proliferative effects, our study is expected to provide clues for a better comprehension of the AR-dependent inhibition of estrogen-related cancer cell proliferation.

**NAMT18. Effects of New Compounds from Marine Sources in Human Colorectal Cancer Cell Lines**

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**Background:** Several agents originating from marine sources, both plants and animals, have shown anticancer activities. To date, at least 40 compounds isolated from marine sponges have shown anticancer effects, mainly pro-apoptotic, in human. **Methods:** In this preliminary study, we tested the effects of new compounds from marine microorganisms cultured by SZN of Naples and extracted by the Institute of Biomolecular Chemistry ICB, CNR, Naples. In particular, we evaluated the potential antiproliferative effects of five extracts from marine source (MC1, MC2, MC3 MC4 and MC5) in DLD1 and SW620 human colorectal cancer cell (CRC) lines, through MTT and BrdU incorporation assays. **Results:** Results showed that the 24 h and 48h-treatment of DLD1 and SW620 cells with MC5 at concentrations from 1  $\mu$ M significantly inhibited cell proliferation that was reduced of about 20% by comparison with untreated CRC cells. For MC5 doses higher than 5 $\mu$ M strongly inhibited colon cancer cell proliferation inducing a percentage of inhibition higher than 90%. Treatment with MC3 10 $\mu$ M significantly reduced the proliferation after 48h of treatment in both cell lines. Finally, the extract MC2 used at doses higher than 5  $\mu$ M

significantly reduced the proliferation in DLD1 but not in SW620. **Conclusions:** Obtained data indicate that MC5, MC3 and MC2 extracts interfere with CRC cell proliferation in a dose- and time-dependent manner. Although further research needs to be focused on clarifying the pathways induced by the observed antiproliferative effects, the results showed that the extracts from marine source evaluated in this study are promising for development of new anticancer drugs.

#### NEOPLASIA – CANCER STEM CELLS

##### NSC1. CD133 Protein Regulates Cell Proliferation, Tumorigenicity and Drug Resistance in Human Colon Cancer Cells

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**Background:** CD133 is a transmembrane pentaspan molecule considered a putative stem cell marker in several normal and cancer tissues. Surface expression of CD133 identifies a subpopulation of tumor-initiating cells, having the properties of self-renewal, proliferation and multilineage differentiation, in a variety of human cancers, including colon cancer. Although CD133 is considered a useful marker to identify cancer stem cells (CSC), its exact role(s) in human colorectal tumorigenesis remain unknown. **Methods:** We found that positive CD133 staining is an independent predictor of shorter survival in colorectal cancer patients. Thus, to shed light on CD133 function(s) and its involvement in the definition of colon CSC phenotype, HCT116 human colon cancer cells were engineered to stably express an exogenous CD133 cDNA, as confirmed by flow cytometry, quantitative real-time PCR and western blot analyses. **Results:** Increased CD133 expression was associated with an increased anchorage-dependent and independent growth *in vitro*, an increased mobility and invasiveness and an increased tumorigenicity *in vivo*. CD133 were also less prone to differentiate when exposed to sodium butyrate. Gene expression profiling identified several genes differentially expressed between CD133-overexpressing derivatives and control cells, including the multidrug resistance-associated protein 2 (MRP2/ABCC2), which was up-regulated in CD133-overexpressing cells, as confirmed by quantitative real-time PCR, and conferred an increased resistance to antineoplastic drugs. **Conclusions:** These results suggest that, besides its role as a potential CSC marker, CD133 might have an important functional relevance in the definition of colon cancer cell phenotype and might represent a useful molecular target for the development of novel anticancer therapies.

##### NSC2. MicroRNA Profiles in Different Contexts of Sonic Hedgehog Signaling: Neuronal and Cancer Stem Cells

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**Background:** Sonic hedgehog controls behavior of stem cells neuronal and cancer stem cells (NSCs and CSCs) identified in medulloblastoma (MB). Aim of the study is to provide new insight in the molecular regulatory circuits involved in maintenance of stemness with particular regard to microRNAs. **Methods:** To this end high-throughput miRNAs profiles have been performed in NSCs, differentiated NSCs and MB-CSCs. NSCs were isolated from wild type mice neonatal cerebellum. CSCs were isolated from both MB arising in Ptc<sup>-/-</sup> mice and human MB. **Results:** The sequencing analysis includes the determination of both known and unknown microRNAs in NSCs, in differentiated NSCs and in MB-CSCs. The analysis of miRNA profiling in NSCs versus differentiated NSCs has revealed specific pattern of microRNAs expression correlated to different Sonic hedgehog signaling activation context. On the other side we were able to determine microRNA features unique to both normal and cancer stem cells implicated in the establishment and/or maintenance of stemness. **Conclusions:** miRNAs differentially expressed in cancer stem cells versus normal stem cells could be those required only in cancer stem cells potentially necessary to neoplastic transformation. The results of this study provide a broad overview of NSC and CSCs microRNAs.

#### NEOPLASIA – HOST PATHOGEN INTERACTIONS IN CANCER

##### NHP1. Analysis and Characterization of Hepatitis B Virus (HBV) DNA Integration into Chromosomal DNA of Patients with Occult HBV Infection and Hepatocellular Carcinoma.

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**Background:** Hepatitis B virus (HBV) DNA integration into the host genome is an important pro-oncogenic event in chronic HBV infection. There is evidence that viral integration may occur also in HBsAg-negative patients with occult HBV infection (OBI). Aim of this study was to investigate and characterize HBV DNA integration in OBI patients with hepatocellular carcinoma (HCC). **Methods:** Tumour specimens from 65 HCC patients were examined (10 HBsAg-positive, 45 OBI-positive and 10 HBsAg-negative/OBI-negative). HBV integration was investigated by Alu-PCR technique. Molecular characterization of virus-genome junctions was performed by cloning and sequencing. **Results:** Integrated HBV DNA was detected in 31/45 (69%) OBI-positive, 8/10 (80%) HBsAg-positive and 0/10 OBI-negative HCC samples, respectively. In OBI cases, HBV integrants were found both in intergenic (50%) and in intragenic genomic regions (50%). HBV integration frequently targeted genes involved in cell growth and adhesion, angiogenesis and cell signaling (i.e. PI4K2A, ADCY5, SCARB1, DNMT1P1, TMEM107, CD 93). Viral integrants were characterized in 20 cases: HBx gene sequences were found in 12 cases, 4 of whom included viral enhancer-II and basal-core promoter; preS1 region including preS1 promoter was found in 1 case; the carboxy-terminal end of the S region plus a portion of the Pol gene were detected in 2 cases; the preCore region in 1 case; S gene sequences in the remaining 4 cases. **Conclusions:** HBV DNA integration is a frequent finding in HCC from OBI patients. This evidence leads to hypothesize that HBV DNA integration may play a pro-oncogenic role in all HBV-infected cases independently of the HBsAg status.

#### NEOPLASIA – MICROENVIRONMENT-DRIVEN TUMOR PROGRESSION

##### NMTP1. Bone Morphogenetic Protein-14 Regulates TGF- $\beta$ -Dependent Angiogenesis in Breast Carcinoma MCF-7 Cells: *In Vitro* and *In Vivo* Control by Anti-TGF- $\beta$ Peptides

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**Background:** TGF- $\beta$  overproduction by breast cancer cells is one of the main characteristics of the late phase of tumor progression and is implicated in metastasis, tumor growth, angiogenesis and immune response. We investigated the therapeutic efficacy of anti-TGF- $\beta$  peptides in the control of TGF- $\beta$  angiogenesis. **Methods:** We have inserted in human MCF-7 mammary cancer cells a mutated TGF- $\beta$  gene in a tetracycline-repressible vector to obtain conditional expression of mature TGF- $\beta$  in the absence of tetracycline upon transient transfection and have evaluated the efficacy of anti-TGF- $\beta$  peptides in the control of MCF-7  $\beta$ -dependent angiogenesis. **Results:** TGF- $\beta$  overexpression induced in MCF-7 several markers of the epithelial-to-mesenchymal transition. Conditioned-medium of TGF- $\beta$ -transfected MCF-7 stimulated angiogenesis *in vivo* and *in vitro* by subsequent activation of ALK5-SMAD2/3 and ALK1-SMAD1/5 signaling in endothelial cells (EC), as well as SMAD4 nuclear translocation, resulting in overexpression of the pro-angiogenic bone-morphogenetic-protein-14 (BMP14/GDF5). Antibody inhibition and siRNA silencing of BMP14 in TGF- $\beta$ -stimulated EC resulted in impairment of BMP14 expression and of TGF- $\beta$ -dependent urokinase-plasminogen activator receptor (uPAR) overproduction, leading to angiogenesis impairment. Two different TGF- $\beta$  antagonist peptides efficiently inhibited all the angiogenesis-related properties elicited in EC by exogenous and conditionally-expressed TGF- $\beta$  *in vivo* and *in vitro*, including SMAD1/5 phosphorylation, SMAD4 nuclear translocation, BMP14 and uPAR overexpression. Antagonist peptides efficiently inhibited *in vivo* angiogenesis either when co-injected with tumor cells or upon systemic administration. **Conclusions:** These preclinical data provide a basis to support using anti-TGF- $\beta$  peptides as a therapeutic agent for TGF $\beta$ -dependent breast cancer angiogenesis.

**NMTP2. An Unexpected Role of the Organic Cation Transporter OCTN1 in Autophagy May Underlie Genetic Variant Association with IBD and Colorectal Cancer.**

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**Background:** We have recently demonstrated that the L503F form of the cation transporter OCTN1, a variant conferring genetic predisposition to intestinal inflammatory bowel disease (IBD), is hyper-represented in ulcerative colitis patients progressing to colorectal cancer (CRC), and also in young CRC patients without overt IBD. Unfortunately, molecular mechanisms linking OCTN1 and its variants to bowel inflammation and to its malignant progression are unknown. **Methods:** The two OCTN1 variants 503L and 503F were overexpressed in in 293T cells, and endogenous OCTN1 (503L) was knocked down in human monocytic THP-1 cells and colon carcinoma Caco-2 cells. Inflammasome activation/secretion of IL-1 and autophagy, two phenomena largely involved in IBD genetic risk, were examined in these cell populations. In addition, bone marrow macrophages from wild- type and OCTN1 KO mice were purified and analysed. **Results:** Maturation/secretion of IL-1 $\beta$  by 293T cells was significantly increased by OCTN1, with Crohn's associated 503F variant having the strongest effect; conversely, IL-1 $\beta$  release was impaired in OCTN1 depleted THP-1 cells and in OCTN1 KO macrophages. Surprisingly autophagy, monitored by western blotting and LC3A-GFP immunofluorescence, followed the same trend in the different cell lines. Moreover, in 293T cells, OCTN1 colocalized with the lysosomal marker cathepsin B, and inhibition of autophagy by 3-MA reduced the processing of pro-IL-1 to mature IL-1 $\beta$ . **Conclusions:** Taken together these results suggest an unexpected role for OCTN1 in the autophagic control of inflammation. Moreover, since autophagy is often deregulated in cancer cells, our findings may explain OCTN1 association with both IBD-related and sporadic CRC.

**NMTP3. NGAL, a NF- $\kappa$ B-Regulated Gene, Is a Chemotactic Factor in Thyroid Cancer**

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**Background:** NF- $\kappa$ B plays an important role in thyroid cancer. In fact, the inhibition of its activity determines the block of the oncogenic potential of anaplastic thyroid carcinoma (ATC) cells. NGAL, a gene under NF- $\kappa$ B transcriptional control, is an acute phase protein able to mediate some of the pro-tumorigenic functions of NF- $\kappa$ B in thyroid cancer, such as the resistance to apoptosis. **Methods:** Monocyte migration was evaluated by transwell assays. Inflammatory infiltrate in tumor specimens was analyzed by immunohistochemistry. **Results:** In the effort to investigate the role of NGAL in cancer-related inflammation, we analyzed the ability of NGAL, secreted in the conditioned medium of ATC cells, to induce monocyte migration *in vitro*. Conditioned medium from NGAL-null ATC cells showed a marked decrease of its chemotactic activity compared to that from parental cells, suggesting a potentially chemotactic role for NGAL in thyroid cancer microenvironment. To test this hypothesis, we down-regulated NGAL expression in the CT26 mouse colon-carcinoma cell line and inoculated parental and NGAL-null CT26 cells in syngenic mice to analyze the inflammatory infiltrate during tumor development. The immunohistochemical analysis of tumors from injected mice showed that the number of lymphocytes and macrophages in tumors developed by NGAL-null CT26 mice was strongly reduced respect to that of parental CT26 mice. **Conclusions:** These data suggest that NGAL secretion by cancer cells could serve as chemoattractant factor for inflammatory cells in tumor microenvironment.

**NMTP4. Mechanism of Action of Metformin as a Potential Angiopreventive Compound**

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**Background:** A significantly decreased risk for cancer in diabetics taking metformin, an antidiabetic drug, has been observed. Inhibiting angiogenesis is a key and common effect of many agents able to repress cancer in pre-clinical and clinical studies and appears to be a key strategy for cancer prevention. However, conflicting data concerning the anti-angiogenic action of metformin have been reported and mechanistic aspects remain to be addressed. **Methods:** We investigated the effects of metformin on angiogenesis *in vivo* with the matrigel sponge assay and *in vitro* using human umbilical vein endothelial cells (HUVEC) to verify the capability of

metformin to interfere with endothelial proliferation, cell death, migration and invasion. **Results:** Our data clearly show that *in vivo* metformin inhibited VEGF induced angiogenesis. To examine the mechanisms underlying this activity, we found that metformin inhibited endothelial cell proliferation without inducing apoptosis by exerting effects on key determinates of cell cycle regulation. Metformin inhibited endothelial cell invasion and repressed the ability of HUVE cells to organize into capillary-like networks in the presence of angiogenic stimuli. These effects were largely reverted by compound C, a specific inhibitor of AMPK, suggesting that the mechanism involves activation of AMPK signaling. Gene expression profiles of endothelial cells treated with metformin showed down-regulation of several angiogenesis-related genes. **Conclusions:** Taken together, our results show a clear anti-angiogenic action of metformin that appears to act directly on endothelial cells that may in part explain the reduced tumor incidence in patients on metformin.

**NEOPLASIA – NOVEL BIOMARKERS IN ONCOLOGY**

**NBM1. Molecular and Functional Characterization of Annexin A3 in Human Normal Cortex and Renal Cell Carcinoma Primary Cultures**

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**Background:** Annexin A3 (AnxA3) is a member of the annexin protein family involved in the regulation of membrane trafficking, ion channels and proliferation. The role of AnxA3 in kidney and in renal carcinomas is still unclear, even if it has been described its possible involvement in the enhancement of the transactivating activity of HIF-1 $\alpha$ , involved in the pathogenesis of renal carcinomas (RCC) and constitutively expressed in 80% of clear cell RCC because of the biallelic inactivation of VHL. **Methods:** For molecular and functional characterization of AnxA3 in normal kidney and RCC, we used primary cell cultures established in our laboratory and submitted to gene silencing, immunofluorescence and western blot analysis. **Results:** Normal cortex and RCC primary cultures show a differential expression pattern, correlated with HIF-1 $\alpha$  level, of two AnxA3 isoforms of 33 and 36 kDa. Moreover, total AnxA3 protein level results down-regulated in RCC respect to cortex cultures. siRNAs specific to 36kDa AnxA3 isoform induced a down-regulation of Lysyl oxidase transcript level, a direct target of HIF-1 $\alpha$ , in cortex and HIF-1 $\alpha$  negative RCC primary cultures. Immunofluorescence analysis evidenced in normal cells a cytoplasmic vesicular distribution of AnxA3, particularly in the perinuclear area, and also inside the nucleus. Instead in HIF-1 $\alpha$  positive RCC cells AnxA3 showed only a vesicular cytoplasmic signal, as confirmed by western blot analysis on purified subcellular fractions. Moreover, AnxA3 colocalized in cytoplasm with endosomal markers. **Conclusions:** The different subcellular distribution of AnxA3 in normal and RCC cells might differently modulate HIF-1 $\alpha$  nuclear uptake and its transactivating activity.

**NBM2. Kaiso, a Key Regulator in EMT and Cancer Progression**

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**Background:** Advanced stages of cancer are characterized by increased aggressiveness, invasiveness, and the down-regulation of tumor suppressor genes via methylation. Kaiso, a bimodal transcription factor, interacts with DNA through either a DNA consensus sequence or methylated CpG dinucleotides, thus regulating gene expression. A clinical role for Kaiso expression in advanced stages of breast and prostate cancer remain unclear. Here we hypothesize that there will be a correlation between Kaiso expression, localization, and breast and prostate cancer progression. **Methods:** Immunohistochemistry was performed to examine protein expression and localization human breast and prostate tissue. Kaiso-specific siRNA/small hairpin (sh) RNA was used to decrease Kaiso expression levels in MDA-MB-231, MDA-MB-468, DU-145, DU 145WT, PC3 cell lines. Subsequently, mRNA, protein and localization were measured using qRT-PCR, western Blot and immunofluorescence. Cell motility and invasion was assessed in the siRNA/sh-RNA Kaiso transfected cells. **Results:** An overall increase in Kaiso expression was found in primary tumor samples and this increased as cancer progressed to distant sites. Additionally, nuclear localization of Kaiso was observed in primary tumor and lymph node metastasis. siRNA/sh-RNA Kaiso treated cell lines showed a delay in cell motility, invasion. 5-aza-2'-deoxycytidine treated cells showed a re-expression of tumor suppressor E-cadherin, which correlated with E-cadherin re-expression in siRNA/sh-RNA Kaiso treated cells. **Conclusions:** Down-regulation of E-cadherin, migration and invasion are key to cancer progression and metastasis. The ability of

Kaiso to regulate these key characteristics of cancer progression suggests it is a relevant therapeutic target and a potential indicator of breast and prostate cancer.

**NBM3. Blood Cholesterol and Sphingomyelin in Patients with Cancer**

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**Background:** It is generally believed that high levels of blood cholesterol are harmful to health, whereas low levels seem to be positive and therefore are often neglected. Unfortunately this assumption is far from true, since severe hypocholesterolemia can be indicative of cancer. Cancer cells avidly incorporate serum cholesterol, favouring the expression of proteins involved in cell proliferation such as RNA polymerase II, STAT3, PKC $\alpha$  and cyclin D1 (Pugliese et al. Eur. J. Cancer 46:1735). Numerous studies have shown that a strong interaction exists between unesterified cholesterol and saturated fatty acid sphingomyelin, which arises from the Van der Waals interaction. Since sphingomyelin and cholesterol association is responsible for formation of membrane lipid rafts involved in cell signalling we have studied the possible hyposphingomyelinemia associated to hypocholesterolemia in patients with cancer. **Methods:** The blood of 25 patients with monoclonal gammopathy were analyzed for their proteins and lipids content. **Results:** The results demonstrated that the patients with high level of gamma proteins present a strong decrease of both cholesterol and sphingomyelin content in blood. **Conclusions:** The results suggested the possible incorporation of cholesterol-sphingomyelin nano-sized vesicles that could change the structure/function of cell membrane or intracellular virtosomes. To investigate the reason for increased lipid incorporation by tumor cells, we performed lipidomic study on the serum of the same patients. Initial results show a lipid profile different from that of healthy subjects.

**NBM4. B-Lymphocyte Stimulator Versus Chromogranin A in the Follow-Up of Patients with Neuroendocrine Tumors**

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**Background:** Chromogranin A (CgA) is the most recommended marker in neuroendocrine tumors (NET), but generally presents low sensitivity and specificity. We recently (Fabris et al. Immun Endoc & Metab Agents in Med Chem 2011) described high levels of B-lymphocyte stimulator (BLYS) in patients with neuroendocrine tumors (NET). Here we assessed BLYS role in the follow-up of NET patients compared to CgA. **Methods:** We enrolled 109 NET patients (25.7% lungs, 74.3% gastro-enteropancreatics, 56% low grade, 44% high grade), 66.7% with pathological CgA serum levels and 37.6% metastatic. Patients were classified in 3 subgroups: in remission (19), with evidence of persistent but stable disease (42), relapsing or further progressive disease (48). BLYS and CgA were analyzed by ELISA, in 41 patients also after 8  $\pm$  6 months. **Results:** We confirmed BLYS up-regulation in NETs, 65% presenting pathological levels. BLYS did not correlate significantly with CgA. Progressive patients presented higher BLYS than stable and remission ( $P < 0.0001$ ). Compared to CgA, BLYS appeared slightly less specific (high BLYS in 29.4% vs. CgA pathological in 17.6% of remission cases;  $P = ns$ ), but significantly more sensible for progression (high BLYS in 87.5% vs. CgA pathological in 68.8% of progressive patients;  $P = 0.019$ ). In CgA-negative cases, 86.7% of progressive patients presented high BLYS levels compared to 28.6% of remission cases ( $P = 0.0025$ ). In the follow-up, BLYS remained unchanged in stable patients, decreased in improving patients ( $P = 0.0078$ ) and increased significantly in relapsing ( $P = 0.03$ ). CgA did not change significantly in relapsing. **Conclusions:** BLYS appears as a new useful marker in the follow-up of NET patients.

**NBM5. Gelatinolytic Activities in the Sera and in the Urine from Patients with Prostate Diseases**

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**Background:** It is widely recognized that the serum prostate-specific antigen (PSA) as a biomarker of prostate cancer is imperfect, in that it can have many false positive elevations attributable to benign hyperplasia and subclinical prostatic inflammation. There are increasing data that support a positive correlation between gelatinase (MMP-2 and-9) activity and tumor cell invasion and tumor aggressiveness. **Methods:** By gelatin zymography we verified MMP activity in the sera and in the concentrate urine of patients with prostate disease. Of these patients, 30 had cancer, consisting of 13 Gleason score 6, twelve Gleason 7, two Gleason 8, three Gleason 9, and 8 had benign prostate hyperplasia (BPH). **Results:** Four dominant gelatinolytic bands were detected migrating at ~ 240, 130, 92 and 72 kDa. The most abundant lytic activity is at 92 kDa (MMP-9); whereas MMP-2 is present in lesser quantities. MMP-9 activity is enhanced in the sera from patients with cancer compared with BPH

patients. On the contrary in the urine specimens, MMP-9 activity is enhanced in the patients with BPH compared with cancer patients. No correlation between gelatinolytic activity and Gleason score or pathological findings was found.

**Conclusions:** These results suggest that the inexpensive measurement of MMPs may serve as a suitable supplementary tool to distinguish between patients with prostate cancer and patients with BPH, and the addition of this enzyme to currently available PSA and/or f-PSA/t-PSA ratio might provide clinicians additional objective information on prostate carcinomas.

**NBM6. PGE2 Induces Epigenetic Modifications and Up-Regulation of IL-8 Gene in Astrocytoma**

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**Background:** It is now well recognized that chronic inflammation is a risk factor for cancer. Several pro-inflammatory gene products, among which interleukin-8 (IL-8), have been linked to tumorigenesis, promotion, proliferation, invasion, angiogenesis, and metastasis. We previously reported that astrocytomas express high levels of this chemokine in response to an increased synthesis of prostaglandin E2 (PGE2). Here, we investigated whether the PGE2-induced IL-8 activation is mediated by epigenetic modifications. **Methods:** DNA methylation status of the 6 CpG sites within IL-8 promoter region and histone acetylation levels were analyzed in two astrocytoma cell lines of different malignancy grade and normal astrocytic cells by bisulphite sequencing and chromatin immunoprecipitation, respectively. IL-8 mRNA was quantized by real-time PCR and protein levels were measured by enzyme immunoassay. **Results:** PGE2 activated IL-8 transcription through specific demethylation of an individual CpG residue (nucleotide -83) located within the C/EBP-beta consensus sequence in the IL-8 promoter and abnormal acetylation of histone H3 in this region of chromatin. These events promoted the recruitment of C/EBP-beta transcription factor which, in turn, formed a docking platform for p300 cofactor, leading ultimately to enhanced transcriptional potential of IL-8.

**Conclusions:** Our findings have elucidated an orchestrated mechanism triggered by PGE2 whereby concurrent association of site-specific demethylation and histone H3 hyperacetylation resulted in derepression of IL-8 gene expression in astrocytomas. These observations imply that anti-inflammatory agents that suppress IL-8 or IL-8-regulated products should have a potential in both the prevention and treatment of this cancer.

**NBM7. Polymorphisms of Genes of TGF- $\beta$  Pathway and Susceptibility to Colorectal Cancer**

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**Background:** Genetic background implicated in cytokine network may have a key role in the susceptibility to colorectal cancer (CRC). The TGF- $\beta$  pathway is involved in several biological processes, including cell proliferation, differentiation, migration and apoptosis. **Methods:** rs1800471 SNP polymorphism of TGF- $\beta$ 1 rs334348 and rs334349 of TGF- $\beta$ R1, rs900 of TGF- $\beta$ 2 and rs4522809 of TGF- $\beta$ 2R2 were typed in a group of 82 patients affected by sporadic CRC and in 237 age- and sex-matched healthy controls, using a competitive allele specific PCR assays (KASPar), developed by KBioscience (England). **Results:** No significant genetic contribution has been observed for 3 of the 5 SNPs tested. Indeed, a significant different allelic distribution between patients and controls has been observed for the polymorphism G $\rightarrow$ C (rs1800471) responsible for an arginine vs. proline missense change (R25P) in codon 25 of the TGF- $\beta$  gene ( $P = 0.021$ ). By this analysis, a weak protective role would emerge for the minor allele C in the susceptibility to the disease. Furthermore the analysis of genotype and allelic frequencies of rs4522809 showed a statistically significant difference ( $p = 0.0016$  and  $P = 0.0019$  respectively) between patients and controls. **Conclusions:** All together these results, suggest that functional relevant SNPs of TGF-beta pathway might be involved in susceptibility to CRC, influencing the extension and severity of the disease.

**NBM8. Differential Expression Profiling of MicroRNAs in Human Cutaneous and Uveal Melanoma**

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**Background:** Over the past decade, microRNA (miRNA)-mediated epigenetic regulation of tumour suppressor genes and oncogenes has been shown to play a central role in melanomagenesis. Here, we examined the miRNA signature discriminating cutaneous and uveal melanoma. **Methods:** Genome-wide profiling of miRNAs was performed in cutaneous and uveal melanoma cell lines by human miRNA microarray platform (Agilent Sanger miRBase-release 10.1) with 723 human

and 76 human viral miRNAs represented. Agilent Feature Extraction Software was used for background subtraction. LOWESS and Quantile normalizations were performed. miRNA microarray expression data were validated by RT-PCR. **Results:** Relative to normal melanocytes, in uveal melanoma cells, miR-130b, miR-193b, miR-320a, and miR-9\* significantly decreased, and miR-654-3p markedly increased; in cutaneous melanoma cells, miR-199a-3p and miR-22 were down-regulated, whereas let-7g was up-regulated. Two of these miRNAs, miR-193b and let-7g, were previously shown as potential regulators in melanoma, whereas the other ones have not been related to melanoma yet. **Conclusions:** Our analysis enables us to identify miRNAs that have not previously been associated with melanoma. In addition, the comprehensive survey of differentially expressed miRNAs shows remarkable differences between cutaneous and uveal melanoma. Although the study is preliminary, we believe the results add to the present knowledge on miRNA dysregulation in melanoma carcinogenesis. As such the results would serve as a starting point for identifying the direct targets of key miRNAs and elucidating their mechanisms of regulation. Understanding the functional roles of miRNAs in melanoma will contribute to the development of targeted therapy.

**NBM9. Higher Frequency of Hypermethylation of p16INK4A Compared to p14ARF among Cutaneous Melanoma Patients from Southern Italy**

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**Background:** Epigenetic deregulation due to p14ARF and p16INK4A promoter hypermethylation has been previously reported in many cancers, including cutaneous melanoma (CM). Nevertheless, discriminating the involvement of these genes in CM still remains an open question. **Methods:** p14ARF and p16INK4A were analyzed in 60 CM formalin-fixed paraffin-embedded tissue sections and in G-361 and GR-M cutaneous melanoma cell lines by methylation-specific PCR and sequencing. Gene expression was evaluated by qReal-time PCR. Cell lines were treated with demethylating agent 5-aza-2'-deoxycytidine. **Results:** p16INK4A gene promoter methylation was found in 36 of 60 (60%) melanoma tissues, 12 of which were heterozygous and the others homozygous. Conversely, p14ARF was found methylated in heterozygous status in 19/60 (31.67%) cases. Hypermethylation of both genes showed low frequency (10%). G-361 and GR-M cell lines were identified as homozygous methylated in p16INK4A and unmethylated in p14ARF. Loss and decrease gene expression was observed in homozygous and heterozygous status, respectively. All cells exhibited demethylation and induction of gene expression after 5-aza-dC treatment. **Conclusions:** Here we showed a higher frequency of methylated CpGs in p16INK4A compared to p14ARF in cutaneous melanoma, unlike other studies that have reported the opposite. As this is the first detailed investigation of the frequency of p16INK4A and p14ARF methylation in CM in Southern Italy, it is likely that the well-known high occurrence of melanoma in this region may be associated to aberrant methylation of p16INK4A. To note, CM tissue analysis facilitates a heterogeneity of results not otherwise demonstrable in cell lines.

**NBM10. A New High-Speed Nested PCR-RFLP Method for the Screening Of V600E BRAF Mutation in Thyroid Tissue and Cytological Samples**

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**Background:** Recent studies have shown that BRAF activation point mutations are present in about 45% (range 29-69%) of papillary thyroid cancers (PTC). Almost 90% of the mutations are BRAF T1799A transversion in exon 15 which results in a (V600E) substitution. Fine needle aspiration biopsy (FNAB) is the primary tool to distinguish benign from malignant nodules. The aim of this study is to establish an accurate and sensitive molecular method to detect BRAF V600E mutation as a biomarker of early PTC. **Methods:** We analyzed the genomic DNA extracted from 100 PTC patients, previously characterized by cytological examination. BRAF V600E mutation was evaluated by RFLP-PCR, using an artificial HpyCH4-IV restriction site in the PCR product, corresponding to V600E mutation. Our method detected heterozygous V600E BRAF mutation in 40% of PTC samples analyzed. **Results:** All samples analyzed in heterozygous showed a panel of three restriction fragments (147, 126 and 21 bp), whereas the wild-type samples showed a pattern of two restriction fragments (126 and 21 bp). The results of sequencing are overlapping up to a concentration of pathological cells of 35%, whereas our nested PCR-RFLP method was able to discriminate to a sensitivity of 1% pathological cells.

**Conclusions:** The validation of the method of analysis performed on all samples by direct sequencing enhances the precision, accuracy, specificity and sensitivity of both the method and results. These data are indicative for an innovative and

sensitive technique for the evaluation of the BRAF V600E mutation and can be a useful tool for screening BRAF V600E mutations.

**NEOPLASIA – TUMOR IMMUNITY**

**NTIM1. Immunogenic Dendritic Cell Selection by Natural Killer Cells During Anti-Cancer Immune Response**

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**Background:** Previous studies have reported that activated natural killer (NK) cells can kill autologous immature dendritic cells (DCs) *in vitro*, whereas they spare fully activated DCs. This led to the proposal that activated NK cells might select a more immunogenic subset of DCs during a protective immune response. However, there is no demonstration that autologous DC killing by NK cells is an event occurring *in vivo* and, consequently, the functional relevance of this killing remains elusive. **Methods:** NK cells were activated in a mouse model by injecting MHC-devoid cells. Draining lymph nodes were collected and lymph node DC functions analyzed. Finally, in a model of anti-cancer vaccination, the functional relevance of DC editing by NK cells was investigated. **Results:** A significant decrease of CD11c+ DCs was observed in draining lymphnodes of mice inoculated with MHC-devoid cells. Residual lymph node DCs displayed an improved capability to induce T cell proliferation. In addition, during anti-cancer vaccination, the administration of MHC-devoid cells together with tumor cells increased the number of tumor-specific CTLs and resulted in a significant increase in survival of mice upon challenge with a lethal dose of tumor cells. Depletion of NK cells or the use of perforin knockout mice strongly decreased the tumor-specific CTL expansion and its protective role against tumor cell challenge. **Conclusions:** Our data support the hypothesis that NK cell-mediated DC killing takes place *in vivo* and is able to promote expansion of cancer-specific CTLs. Our results also indicate that cancer vaccines could be improved by strategies aimed at activating NK cells.

**NTIM2. Gene Regulation by microRNA Is Associated with CD8+ T Cells Immune Deviation in Renal Cell Carcinoma Patients: Role of JAK3 and MCL-1**

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**Background:** MicroRNAs (miRNAs) are important regulators of gene expression and numerous miRNAs are expressed aberrantly and correlate with tumorigenesis. In patients with renal cell carcinoma (RCC), T cell immune dysfunctions have been reported. The aim of our study was to assess gene expression profiles and their regulatory mechanisms by miRNAs on CD8+ T cells from RCC patients, at basal (day 0) and after stimulation against RCC line (day 35). **Methods:** We compared autologous and allogeneic CD8+ T-cell responses against RCC line generated from RCC patients and their HLA-matched healthy donors. We then analyzed the gene expression profiles of CD8+ T cells by microarray approach and then identified molecular mechanisms of gene regulation by miRNAs analysis. **Results:** Comparison of gene expression data in allogeneic CD8+ T cell vs autologous RCC-reactive CD8+ T cells demonstrated differential expression of genes involved in apoptosis and regulation of cell proliferation. Among these genes, the down-regulation of JAK3 and MCL-1 gene expression in patient CD8+ T cells versus their healthy counterparts was observed. We found evidence for defective suppressor activity of miR-29b and miR-198 in regulating gene expression of JAK3 and MCL-1 in RCC CD8+ T cells. Transfection experiments on isolated PBMCs from RCC patients using anti-hsa-miR-29b and anti-hsa-miR-198 inhibitors revealed a significant up-regulation of both proteins and a significant improvement of cell survival *in vitro*. **Conclusions:** miR-29b and miR-198 play a key role in regulating immune-mediated mechanisms by interfering in CD8+ T cells gene expression of JAK3 and MCL-1 and may have important therapeutic implications.

**NTIM3. CD40 Cross-Linking Induces Migration of Renal Tumor Cell Through NFAT Activation and Integrin  $\beta$ 1 Reorganization**

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**Background:** CD40 crosslinking play an important role in regulating cell migration, adhesion and proliferation in renal cell carcinoma (RCC). CD40/CD40L interaction on RCC cells activates different intracellular pathways, however the molecular mechanisms leading to cell scattering are not clearly defined. Aim of our study was

to investigate the principal intracellular factors activated by CD40 ligation and their specific involvement in RCC cell migration. **Methods:** RCC cell lines were isolated from kidney tissue samples of patients affected by RCC and subsequently stimulated with CD40L. **Results:** We found that CD40-CD40L interaction induced cell proliferation through a cytoskeleton reorganization and integrin  $\beta 1$  distribution, whereas it did not affect apoptosis. Interestingly, CD40 ligation did not activate the pathway involving phosphatidylinositol 3-kinase (PI3K), Akt and p70 ribosomal S6 kinase, but it increased the phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun NH(2)-terminal kinase (JNK) and p38 MAPK. Furthermore, CD40 crosslinking activated different transcriptional factors on RCC cell lines: AP-1, NF- $\kappa$ B and some members of the NFATs family. In particular, the specific inhibition of NFAT factors by cyclosporine A, completely blocked RCC cell motility induced by CD40 ligation. **Conclusions:** These findings support the hypothesis that CD40 ligation induces cell scattering through cytoskeleton reorganization and activation of different intracellular signalling pathways, in particular the NFATs family. These factors could represent a potential therapeutic target in the setting of patients with metastatic RCC.

## NEOPLASIA – TUMOR PROGRESSION

### NTP1. Estrogen Induces Looping Between Tumor Suppressor *RIZ* Gene Promoter 2 with Exon 9a

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**Background:** The dynamic intra- and inter-chromosomal links between specific loci contribute to the creation of cell type-specific gene expression profiles and to gene regulation during differentiation processes. Looping is implicated in bringing together far upstream or downstream regions with the gene promoter and body sites, and in establishing contacts between the 5' and 3' ends of genes, since 3' end-processing factors interact with components of the transcriptional machinery. The tumor suppressor PRDM2/*RIZ* gene plays a role in controlling cellular processes, such as cell cycle progression and regulation of development. The retinoblastoma protein-interacting zinc-finger gene (*RIZ*) is estrogen responsive and has two alternative promoters, the more downstream of which, promoter 2, is nearby to an ERE-sequence and is involved in estrogen receptor transcriptional activation. **Methods:** With the innovative DNA-Picked Chromatin (DPC) assay after timecourse of 17- $\beta$ -estradiol (E2) induction of MCF-7 breast cancer cells, we highlight preferential interaction between hormone-responsive *RIZ* promoter and the polyadenylation sites. Gene expression analysis of induced cell RNA was performed with qRT-PCR assay. **Results:** Within 60' of E2 treatment of cells, we have observed increased exon segments, exons 9a and 10 (alternative polyA site), linked to isolated promoter 2 and concomitant decrease of exon10 to *RIZ* promoter 1. The exon 9a shows a low association to *RIZ* promoter 1 without E2. qRT-PCR also demonstrated increased exon 9a-containing transcripts. **Conclusions:** The E2 remodels the chromatin architecture of PRDM2/*RIZ* gene locus to create a loop for the mRNA transcription with polyA-exon 9a, leading to the production of oncogenic variants.

### NTP2. Role of ER- $\alpha$ in the Modulatory Effect of Adiponectin on Breast Cancer Cell Growth

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**Background:** Several studies suggest that adiponectin, a hormone mainly produced by adipose tissue, may influence cancer pathogenesis. Circulating adiponectin levels appear to be inversely associated with an increased risk of breast cancer. **Methods:** MTT assay, immunolocalization, RT-PCR, transient transfection, ChIP assay. **Results:** We demonstrated that adiponectin inhibited proliferation in ER- $\alpha$ -negative cells, whereas it stimulated growth in ER- $\alpha$ -positive cells. Adiponectin is able to reproduce the classic features of ER- $\alpha$  transactivation in MCF-7 cells: nuclear localization, down-regulation of its mRNA and protein levels, and up-regulation of the estrogen-dependent genes, cathepsin D and pS2. In MCF-7 cells, adiponectin up-regulated mRNA and protein levels of cyclin D1 (CD1), which, in contrast, appeared down-regulated in MDA-MB-231 cells. Similar opposite effects were elicited by adiponectin on CD1 promoter activity. Mutagenesis studies revealed that the modulation of CD1 promoter activity by adiponectin was mediated mainly by the Sp1 motif. Moreover, adiponectin induced Sp1 nuclear localization and its phosphorylation. To provide insight into the molecular mechanism by which the Sp1 motif modulates CD1 promoter activity, we performed ChIP experiments. In MCF-7 cells adiponectin increased Sp1/ER- $\alpha$  complex, enhanced Pol-II and pCAF recruitment, addressing the involvement of an activator complex that mediated the adiponectin-induced transcriptional activation of CD1. In contrast, in MDA-MB-231

cells adiponectin recruited Sp1, displaced Pol-II and recruited a corepressor complex containing SMRT, NCoR and HDAC1. **Conclusions:** Thus, on the basis of our findings we suggest that a proper use of novel therapeutic tools potentiating adiponectin signaling in breast cancer may target ER- $\alpha$ -negative tumor growth and progression.

### NTP3. Estrogen Receptor- $\beta$ , through Sp1, Recruits a Corepressor Complex to the Estrogen Receptor- $\alpha$ Gene Promoter in Breast Cancer Cells

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**Background:** In the regulation of mammary gland growth and development human estrogen receptors (ERs)  $\alpha$  and  $\beta$  play a crucial role. Normal breast tissues display a relative higher expression of ER- $\beta$  than ER- $\alpha$ , which drastically changes during breast tumorigenesis. The different ratio of expression of the two proteins may be involved in breast carcinogenesis development. However, the molecular mechanisms underlying the potential opposing roles played by the two estrogen receptors on tumor cell growth remain to be elucidated. **Methods:** Transient transfection, Western Blotting, EMSA, ChIP, RT PCR, silencing. **Results:** In this study, we have showed that ER- $\beta$  overexpression in breast cancer cells decreases cell proliferation and down-regulates ER- $\alpha$  mRNA and protein content, along with a concomitant repression of estrogen-regulated genes. Deletion analysis of the human ER- $\alpha$  promoter region, indicated that elevated levels of ER- $\beta$  down-regulated basal ER- $\alpha$  promoter activity. Furthermore, site-directed mutagenesis revealed that the proximal GC-rich motifs at -223 and -214 are critical for the ER- $\beta$ -induced ER- $\alpha$  down-regulation in breast cancer cells. The results indicate an enhancement recruitment of Sp1 and ER- $\beta$ , together with the corepressor NCoR, to the ER- $\alpha$  promoter region concomitant with the hypoacetylation of histone H4 and displacement of RNA polymerase II. Silencing of NCoR gene expression by RNA interference reversed the down-regulatory effects of ER- $\beta$  on ER- $\alpha$  gene expression and cell proliferation. **Conclusions:** Our data suggest a novel mechanism by which overexpression of ER- $\beta$  through NCoR is able to down regulate ER- $\alpha$  gene expression, thus inhibiting the driving role of ER- $\alpha$  on breast cancer cell growth.

### NTP4. Role of Argonaute 2 in Estrogen Receptor- $\beta$ -Mediated Transcriptional Gene Silencing in Breast Cancer Cells

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**Background:** Estrogen receptor (ER) is the primary target for chemoprevention and endocrine therapy in breast cancer and provides prognostic and predictive information about tumor response to endocrine therapies. Expression of ER- $\beta$  reduces cancer cell proliferation and tumor growth, suggesting an anti-proliferative and a positive prognostic value of this receptor subtype. Although ER- $\beta$  seems to be a tumor suppressor, its role in human breast carcinogenesis remains to be elucidated. ER- $\alpha$  and ER- $\beta$  could show the ability to interact with the same proteins resulting, however, in divergent transcriptional effects. Moreover, it has emerged that unliganded ER- $\beta$  exhibits an active role in constitutive regulation of target genes transcription. **Methods:** Tandem affinity purification (TAP) and mass spectrometry (MS) were applied to identify unliganded ER- $\beta$  nuclear interacting proteins. Among the over 300 partners identified we investigated the functional significance of ER- $\beta$ -AGO2, since this last protein has been directly implicated in transcriptional gene silencing (TGS) induced by microRNAs. Co-immunoprecipitation assays, confocal microscopy (Proximity Ligation Assay/PLA) ChIP-Seq, siRNA-mediated gene knock-down (KD) and gene expression profiling were applied to this end. **Results:** Co-immunoprecipitation and PLA confirmed ER- $\beta$ /AGO2 association in MCF-7 cell clones expressing tagged ER- $\beta$ . Co-immunoprecipitation was also observed for several known AGO2 interacting proteins, identified by TAP/MS as ER- $\beta$  partners. ChIP-Seq allowed the identification of a large number of ER- $\beta$ - and AGO-binding sites and the corresponding target genes, whereas AGO2 KD resulted in significant changes in gene expression profiles. **Conclusions:** Experimental evidences suggest that AGO2 is a partner of ER- $\beta$  involved in gene regulation in hormone responsive breast cancer cells. Supported by AIRC; MIUR; Regione Campania; University of Salerno; Fondazione con il Sud; EU COST Action BM1006 and 'SeqAhead' FEBS Short Term Fellowship to G.Nassa.

**NTP5. Role of AKT-Regulated MicroRNAs in Non-Small Cell Lung Cancer**

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**Background:** MicroRNAs (miRNAs) are small non-coding RNAs usually of 21-25 nucleotides in length that regulate gene expression at post-transcriptional level. miRNA deregulation plays an important role in lung cancer development. The objective of this study is to discover the potential link between deregulation of the PI3K/AKT pathway and miRNA expression in lung cancer. **Methods:** By microarray hybridization we identified the miRNA expression profile in two cellular systems: (1) BEAS-2B, human immortalized, non tumorigenic, lung epithelial cells genetically modified for the activation of the PI3K/AKT pathway through expression of mutant AKT1 (E17K), PI3K (E545K) or silenced for PTEN; and (2) NCI-H460, non small cell lung cancer (NSCLC) cell line genetically modified for the knockdown of the PI3K/AKT pathway through the silencing of AKT1, AKT2 and PI3K. **Results:** Microarray data analysis identified several differentially expressed miRNAs in lentivirus-infected cells compared to the parental cell lines. In particular, miR-196a was significantly up-regulated in BEAS and down-regulated in H460. Subsequently, we observed that miR-196a is overexpressed in human primary lung cancer samples. miR-196a is predicted, using bioinformatic tools, to target genes, such as FoxO1, FoxO3 and p27, involved in the PI3K/AKT pathway. The generation of stable cellular clones which overexpress miR-196a, or silenced for this miRNA through the expression of an anti-miR-196a, is important to determine the role of miR-196a in the development of human lung cancer. By cell migration assays we demonstrated the involvement of miR-196a in the increased ability of cells to migrate. **Conclusions:** Further biological studies are necessary to characterize the role of miR-196a in NSCLC.

**NTP6. Abstract Withdrawn**

**NTP7. Rosiglitazone and AS601245 Decrease Cell Adhesion and Migration Through Modulation of Specific Gene Expression in Human Colon Cancer Cells.**

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**Background:** PPARs are nuclear receptors activated by ligands. Activation of PPAR $\gamma$  leads to a reduction of adhesion and motility in some cancer models. PPAR $\gamma$  transcriptional activity can be negatively regulated by JNK-mediated phosphorylation. We postulated that the use of agents able to inhibit JNK activity, could increase the effectiveness of PPAR $\gamma$  ligands. **Methods:** We analysed the effects of rosiglitazone (PPAR $\gamma$  ligand) and AS601245 (a selective JNK inhibitor) alone or in association, on adhesion and migration of CaCo-2, HT29, and SW480 human colon cancer cells and investigated, through microarray analysis, the genes involved in these processes. **Results:** Cell adhesion and migration was strongly inhibited by rosiglitazone and AS601245. Combined treatment with the two compounds resulted in a greater reduction of the adhesion and migration capacity. Affymetrix analysis in CaCo-2 cells revealed that some genes were highly modulated by the combined treatment, could be involved in these biological responses. Rosiglitazone down-regulated the expression of fibrinogen chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), which were further down-regulated by the combined treatment. Moreover, rosiglitazone, alone or in association with AS601245, caused a decrease in the fibrinogen release. ARHGEF7/ $\beta$ -PIX gene was highly down-regulated by combined treatment, and western blot analysis revealed that  $\beta$ -PIX protein is also down-modulated in CaCo-2, HT29 and SW480 cells. Transfection of CaCo-2 cells with  $\beta$ -PIX gene completely abrogated the inhibitory effect on cell migration, determined by rosiglitazone, AS601245 and combined treatment. **Conclusions:** Results demonstrated that  $\beta$ -PIX protein is involved in the inhibition of cell migration and sustain the positive interaction between PPAR $\gamma$  ligands and anti-inflammatory agents in humans.

**NTP8. The Outcomes of Notch3-Dependent T Cell Leukemia Are Modified by NF- $\kappa$ B Deletion**

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**Background:** The Notch3 deregulation inside T-cell compartment of transgenic (N3-tg) mice, induces an aggressive form of T-cell acute lymphoblastic leukemia (T-ALL), strongly sustained by an NF- $\kappa$ B constitutive activation, mainly represented by the p50/p65-dependent canonical pathway. To clarify the Notch/NF- $\kappa$ B relationships in the onset/progression of T-ALL, we decided to inhibit NF- $\kappa$ B canonical pathway in N3-tg mice. **Methods:** We generated N3-tg/p50<sup>-/-</sup> mice, deleted of the NF- $\kappa$ B/p50 subunit in a Notch3 transgenic background. The follow-up of N3-tg/p50<sup>-/-</sup> versus N3-tg mice was conducted and hematopoietic cell analysis was performed at different ages and in multiple tissues from the indicated animals by flow-cytometry techniques. **Results:** The p50 deletion inhibited the progression of T-ALL in N3-tg/p50<sup>-/-</sup> mice, as defined primarily by the peripheral expansion of immature CD4+CD8+ T cells. Surprisingly, the double mutant mice succumb earlier than N3-tg counterparts. Moribund N3-tg/p50<sup>-/-</sup> mice display the trait of a myeloproliferative disease, with the dramatic expansion of Mac1+Gr1+ myeloid cells in both spleen and blood, as well as of granulocyte/monocyte progenitors in the bone marrow. Preliminary data indicate that these cells do not express Notch3, suggesting that in the absence of p50 expression, Notch3 is able to mainly influence the equilibrium of the myeloid compartment in trans. **Conclusions:** The results presented suggest that the ablation of NF- $\kappa$ B canonical pathway may strongly impact on the outcomes of a T cell specific deregulation of Notch signaling. Thus, providing a useful experimental model to extend our understanding of Notch/NF- $\kappa$ B interplay and to unravel novel strategies for the therapy of different hematological malignancies.

**NTP9. The Resveratrol-Analogue 4,4'-Dihydroxy-Trans-Stilbene Suppresses Transformation in Normal Mouse Fibroblast and Inhibits Proliferation and Invasion of Human Breast Cancer Cells**

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**Background:** 4,4'-dihydroxy-trans-stilbene (DHS) is a synthetic analogue of resveratrol (RSV). We previously demonstrated that DHS exerts a higher antiproliferative activity than RSV on normal human fibroblasts. Herein, our aim was to investigate the effect of DHS both on transformation of BALB/c 3T3 mouse fibroblasts and on proliferation and invasion of human breast cancer MCF-7 cells. **Methods:** Trypan blue staining was used for assessing cell death. Chemically induced (MNNG+TPA) transformation of BALB/c 3T3 mouse fibroblasts was performed with the CytoSelect 96-well cell transformation assay. MCF-7 cell proliferation was investigated by PI staining, BrdU incorporation, and Western blotting analysis. The Boyden chamber cell migration and invasion, gelatin zymography and wound healing assay were also performed. **Results:** DHS efficiently suppressed the two-stage chemically induced transformation in BALB/c 3T3 cells. It also inhibited with high efficiency both anchorage-dependent and -independent MCF-7 growth. In addition, a reduction in the S-phase cell population, associated with an increase in the p21 and p53 protein levels, and with a strong inhibition of the pRb protein phosphorylation, was evidenced in DHS-treated cells. Furthermore, DHS exerted a strong reduction of the matrix metalloproteinase-2 and -9 activities, concomitantly with a marked reduction of cell-cell and cell-extracellular matrix interaction. **Conclusions:** These results demonstrate that the two 4,4'-hydroxyl groups on the stilbenic backbone make DHS a more active molecule, compared to RSV, in inhibiting neoplastic transformation, cancer cell proliferation and invasion. Further *in vivo* studies to better characterize DHS absorption and metabolism, biosafety, and the mechanisms of its anticancer properties are in progress.

**NTP10. DDB2 Interacts with the Nucleotide Excision Repair Proteins at DNA Damaged Sites**

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**Background:** DDB2 is a 48kDa protein originally identified as a component of the heterodimeric complex UV-DDB, together with DDB1. This complex has affinity for the major cytotoxic-mutagenic types of lesions introduced in DNA by UV irradiation, such as 6-4 photoproducts and cyclobutane pyrimidine dimers. These lesions lead to distortion of DNA, predisposing cells to accumulate mutations increasing susceptibility to cancer. Cells, as a protective mechanism against UV induced DNA damage, utilize nucleotide excision repair process. DDB2 plays an important role in the recognition step of UV-induced DNA damage in non-transcribed regions and it is

mutated in xeroderma pigmentosum (group E) patients. In this study, we analyse the possible interaction between DDB2 and other proteins involved in NER process.

**Methods:** We have studied the localization of DDB2 in HeLa cells transiently transfected with pcDNA3.1-DDB2 construct and then irradiated with UV-C at 30 or 100J/m<sup>2</sup>, respectively, for western blot and immunofluorescence analyses.

Moreover, to study the direct interaction between DDB2 and other NER proteins, solubilised chromatin fractions were immunoprecipitated with DDB2 antibody.

**Results:** Cellular localization of DDB2 was examined 5, 10, 30 min post-UV irradiation: the confocal analysis showed that DDB2 co-localized with DNA repair proteins recruited to DNA-damage sites after local UV-C irradiation. The results obtained by immunoprecipitation techniques demonstrated the interaction and physical association between DDB2 and cullin 4A, XPC, XPG, p21 and PCNA proteins after DNA damage. **Conclusions:** DDB2 protein is recruited at local DNA-damaged sites where it directly interacts with NER proteins.

**NTP11. A Novel Role for DNA Polymerase Eta (Polη) in Regulating the Translesion Synthesis Pathway of DNA Damage Tolerance**

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**Background:** Cellular DNA is constantly exposed to ubiquitous environmental genotoxins, such as UV irradiation in sunlight, that cause DNA damage and predispose to cancer and other diseases. Translesion synthesis (TLS) is a DNA damage-tolerance mechanism that uses specialized DNA polymerases to replicate genotoxin-damaged DNA when conventional polymerases stall. TLS polymerases replicate DNA with relatively low fidelity, and their activity must therefore be tightly regulated. Defects in the TLS pathway cause excessive mutagenesis, as evidenced by the xeroderma pigmentosum variant (XPV) syndrome, in which Poleta, the TLS polymerase DNA polymerase eta (Polη), is non-functional. Lack of Polη, which accurately replicates UV-damaged DNA, results in error-prone replication by inappropriate polymerases, UV-induced mutagenesis, and cancer. TLS polymerases are activated when the DNA replication factor PCNA is mono-ubiquitinated by the E3 ubiquitin ligase Rad18. Although this mono-ubiquitination activates TLS, its regulation is poorly understood. **Methods:** We used *in vitro* and *in vivo* biochemical and imaging techniques to map functional interactions between effector proteins in the TLS pathway. **Results:** We have uncovered a previously unidentified function of Polη in the regulation of TLS. In addition to its polymerase activity, we show that Polη recruits Rad18 to damaged DNA to promote efficient PCNA ubiquitination and activate TLS. **Conclusions:** Whereas tumorigenesis in XPV patients has been thought to stem solely from defective Polη polymerase activity, our results reveal that Polη also has non-catalytic roles that regulate TLS. Our findings suggest that imbalanced Polη expression, regardless of exposure to UV light, could misregulate TLS to promote mutagenesis, thus perturbing genomic stability in previously uncharacterized ways.

**NTP12. Nottingham Prognostic Index and Triple-Negative Breast Cancer**  
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**Background:** The Nottingham Prognostic Index (NPI) classifies breast cancers into three prognostic categories: good prognosis for values <3.4 (85% 15-ys-OS) (group1), moderate prognosis for values 3.41-5.4 (42% 15-ys-OS) (group2), poor prognosis for values >5.4 (13% 15-ys-OS) (group3). Our study evaluates the NPI applicability in women affected by invasive triple-negative breast cancer (TNBC) compared to non-TNBC. **Methods:** A retrospective analysis was performed on patients operated in our Department for invasive breast cancer between January 2002 and December 2007 (follow up ≥5years). Women were divided into three groups based on the three NPI prognostic categories, and TNBCs were compared with non-TNBCs in terms of overall survival. **Results:** During the considered period, 917 women were operated for invasive breast cancer at a mean age of 61.4 years (± 12.58). The histological type was in the majority of cases a ductal invasive cancer 72% (661/917); the most frequent TNM stage was T1cN0M0 (stage I); TNBC prevalence was 8% (71/917). The 5- and 8-ys OS resulted respectively 99% (98-100%) and 97% (95-98%) in group1, 88% (83-93%) and 83% (77-89%) in group2, 69% (59-80%) and 55% (44-70%) in group3 (*P* < 0.05). The 5-ys OS in TNBC and non-TNBC stratified for the NPI categories resulted respectively 94% (87-100%) and 99% (98-100%) in group1 (*P* < 0.05), 85% (71-100%) and 89% (84-94%) in group2 (p0.834), 47% (26-85%) and 73% (63-85%) in group3 (*P* < 0.05). **Conclusions:** Despite its old origin and the limited factors considered, NPI represents a valuable prognostic tool also for TNBC.

**PERSONALIZED MEDICINE**

**PM1. Establishment of a Colon Cancer Biobank and Database among Different Institutions**

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**Background:** In the post-genomic era there is still a gap between the available analytical tools and their impact on human diseases. On cancer pathology, that in the next years is expected to be the first cause of death, there is the major need to fill up this gap. In this context the establishment of biobanks in conjunction with databases containing all clinical/pathological/environmental risk data may help the achieving of this goal. We established a colon cancer biobank and relative database involving different hospitals and university departments. **Methods:** The biological material from colon cancer and normal colon has been collected in different institutions, the S Gerardo hospital and Desio-Vimercate hospital that hospitalize patients of a well-defined territory corresponding to Monza-Brianza province. An online questionnaire was produced to collect data on clinical, pathological, and environmental-risk information and the data were centralized in a single database. Models for patient informed consent and regulations to keep the data anonymous were established. The guidelines of standardized operating procedures to collect and store the materials have been tested and made operative. **Results:** We are collecting 170 colon cancer cases/year in conjunction with clinical data. The biobank is favoring the standardization of clinical-diagnostic procedures in the involved hospitals. **Conclusions:** In three years the biobank will be able to provide material for oncological researches addressed to: i) discover biomarkers or therapeutic targets, ii) personalized medicine, iii) systems biomedicine. This experience will be also extended to other tumor pathologies.

**PM2. Identification of a Point Mutation in SMN1 Gene Causing Spinal Muscular Atrophy: Implications for Genetic Counseling and Prenatal Diagnosis**

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**Background:** Spinal muscular atrophy (SMA) is one of the most common autosomal recessive genetic disorders characterized by progressive symmetric muscle weakness and paralysis. Carrier frequency of SMA is approximately 1/35 and therefore the incidence is 1 in 6000-8000 live births. Childhood disease is subdivided into three clinical forms with type I being the most severe. About 95% of SMA cases are caused by homozygous deletion of the *SMN1* gene or its conversion to highly homologous *SMN2*. The remaining cases are heterozygous compound for a deletion/conversion of one *SMN1* allele and a small intragenic mutation of the other allele. This work reports the case of a couple with two SMA type I affected deceased children and the research of mutation/s that determines SMA in this family. The final purpose is to perform prenatal diagnosis in a further pregnancy of the couple. **Methods:** *SMN1* alleles copy number determination is performed by multiplex Real-Time PCR method. Sequence analysis of exons and exon/intron junctions is performed by means of an automated Sanger sequencing. **Results:** The female of the couple is a *SMN1* deletion carrier whereas the male that carries two *SMN1* copies harbours the rs104893922 point mutation (G>A), already described as associated to a severe SMA type I phenotype. The same point-mutation has been identified in some of the healthy siblings. **Conclusions:** The point mutation identified, together with the more frequent *SMN1* gene deletion, allows genetic counselling, carrier testing and prenatal diagnosis for the couple programming future pregnancy and for all relatives at reproductive risk in the family.

**PM3. Linked Data and Translational Medicine: The Role of ICD-11**

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**Background:** Internet and digital information enable strong interactions within an information ecosystem of researchers, clinical practitioners and other resources and users of biomedical data. Currently the lack of standards for data representation is an obstacle in translational research, making difficult to extract all potential knowledge from data acquired through experiments and data analysis. However, the concept of linked data has recently gained relevance, indicating the practice of publishing structured data that can be interlinked and become more useful. The WHO International Classification of Diseases (ICD) is the world's standard tool to capture health information. **Methods:** An exercise is set up using the content model of ICD-11, adopted for the 11th revision of the classification, in which descriptive

characteristics of classification's categories are linked to underpinning standardized terminologies to define information such as signs and symptoms, morphology, caUSI agents or treatment. **Results:** ICD-11 represents a good example of linked data exercise, leading the way to better data USge and therefore faster exploitation of information collected in translational research. Moreover the ICD-11 update and revision process, based on ontological tools, allows for collaborative web-based editing thus opening to all interested parties the possibility to rapidly update the classification and allowing fast transfer of biomedical discoveries into the classifications used in clinical practice. **Conclusions:** The content model of ICD-11 represents a novel enhancing information transfer in translational medicine, but only a large web-based engagement of users in this domain will determine if the classification will become an effective tool for systematic bench-to-bedside knowledge exchange.

#### REDOX REACTIONS IN HUMAN PATHOPHYSIOLOGY

##### RR1. Membrane Oxidative Damage in Intestinal Caco-2 Cells: Protective Effect of a Wine Phenolic Extract

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**Background:** It has been suggested that the beneficial effects on the cardiovascular system ascribed to mild to moderate wine consumption may also arise from a local action in the gastrointestinal tract, where polyphenols and their metabolites concentrate. We investigated the ability of a red wine phenolic extract, obtained from the most typical and widespread grape variety grown in Sardinia, Cannonau, to exert a direct antioxidant action against the oxidative damage to intestinal mucosa.

**Methods:** The protective action of the extract, obtained through liquid-liquid extraction with ethyl acetate, was evaluated as ability to counteract the loss of epithelial integrity, measured as transepithelial electrical resistance (TER) in differentiated human Caco-2 cell monolayers, following tert-butyl hydroperoxide (TBH) exposure, and through MDA, fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto) production (detected through HPLC analysis). **Results:** TBH treatment showed a significant decrease in Caco-2 TER from the lowest concentration tested (0.5 nM) already after 30 min of incubation. TBH 2.5 mM caused the complete loss of membrane integrity after 120 min of incubation. In Caco-2 cells exposed to TBH 2.5 mM for 120 min, a significantly high level of MDA compared to the non oxidized samples, paralleled by an increase of HP and 7-keto values, was detected, indicating an ongoing lipid peroxidation process. Pretreatment with the extract significantly slowed the decrease in TER and inhibited the increase of oxidation products. **Conclusions:** Our results point out for the first time a direct antioxidant action of the phenolic fraction from the wine Cannonau on enterocytes exposed to oxidizing species.

##### RR2. Immune Responses Against Oxidative Stress-Derived Antigens Contributes to Hepatic Inflammation in Nonalcoholic Steatohepatitis (NASH). S. Sutti<sup>1</sup>, A. Jindal<sup>1</sup>, I. Locatelli<sup>1</sup>, M. Vacciano<sup>1</sup>, C. Bozzola<sup>1</sup>, E. Albano<sup>1</sup>

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**Background:** Nonalcoholic steatohepatitis (NASH) has become the most frequent chronic liver disease in relation to the worldwide increase of overweight and obesity. As NASH is often associated with the presence of circulating antibodies against proteins adducted by lipid peroxidation products, we have investigated the relevance of these immune response in the disease pathogenesis. **Methods:** NASH was induced by feeding C57BL/6 and Balb/c mice for 4 weeks with a methionine-choline deficient (MCD) diet. **Results:** Upon MCD feeding C57BL/6 mice showed more liver injury and increased hepatic expression of pro-inflammatory cyto/chemokines than Balb/c mice. In C57BL/6 mice NASH was also associated with increased prevalence of B- and T-lymphocyte infiltration and higher expression of lymphocyte chemokines CCL5 and CXCL10. Although liver oxidative damage was comparable in the two strains, we observed increased titres of IgG against malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE)-derived antigens only in MCD-fed C57BL/6 mice. Furthermore, only C57BL/6 mice had IgG deposits within the hepatic inflammatory infiltrates. To substantiate the role of immunity in the progression of NASH, Balb/c mice were immunized with MDA-adducted bovine serum albumin (MDA-BSA) and subsequently fed with the MCD diet. MDA-BSA immunization did not cause liver injury or inflammation in control mice. Following MCD diet, MDA-BSA-immunized mice showed higher ALT release, more diffuse lobular inflammation and a two-fold stimulation in TNF- $\alpha$  and IL-12 expression as compared to the naive ones. Moreover, liver mRNAs for fibrosis markers pro-collagen 1 $\alpha$  and TGF- $\beta$  were significantly up-regulated by pre-immunization. **Conclusions:** These results indicate that oxidative stress-driven immunity contributes to hepatic inflammation in NASH.

##### RR3. Oncostatin M Induces Epithelial-to-Mesenchymal Transition and Increased Invasiveness in Hepatic Cancer Cells Through Redox Mechanisms

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**Background:** Oncostatin M (OSM) can orchestrate hypoxia-modulated liver processes (development, regeneration, angiogenesis) contributing to chronic liver disease progression and hepatocellular carcinoma (HCC) development. Accordingly, OSM and its related LIFR $\beta$  (leukemia inhibitory factor receptor  $\beta$ ) subunit are overexpressed in cirrhotic liver. Since OSM can operate through hypoxia-inducible-factors (HIFs) and hypoxia has been reported to induce epithelial-to-mesenchymal transition (EMT), this study has been designed to investigate whether OSM may act as a stimulus able to induce EMT in human hepatic cancer cells. **Methods:** EMT, invasiveness and signal transduction were analysed by morphological, molecular and cell biology techniques in HepG2, HuH7 and Madin-Darby canine kidney (MDCK) cells exposed to human recombinant OSM as well as by immunohistochemistry on liver specimens from HCV cirrhotic patients carrying G1 and G2 HCC. **Results:** OSM induced EMT-related changes in all cells (nuclear translocation of SNAI1/2, E-cadherin down-regulation, overexpression of  $\alpha$ -smooth muscle actin and expression of matrix metalloproteinase-2) within 48-72 hrs and stimulated invasiveness in cancer cells. Data revealed a scenario involving early intracellular generation of reactive oxygen species (ROS), activation of PI-3K, ERK1/2, JNK1/2, p38MAPK and STAT3, and phosphorylation/inactivation of GSK-3 $\beta$ . Cancer cell invasiveness was prevented by inhibiting ERK1/2, PI3K or JNK1/2 or by preventing ROS generation. Finally, OSM was expressed in HCC tumor cells in areas also positive for hypoxia-related antigens. **Conclusions:** OSM, expressed in human HCC and peritumoral cirrhotic tissue, can induce EMT in human hepatic cancer cells and stimulate invasiveness through redox-dependent activation of EMT-related critical kinases and transcription factors.

##### RR4. Xanthine Oxidase Directly and Irreversibly Modifies BH4 Contributing to Endothelial Dysfunction in Ischemic Rat Hearts

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**Background:** BH4 is a cofactor of nitricoxidesynthase activity. Changes in BH4 bioavailability may affect vascular function. BH4 is also a potent hydroxyl radical scavenger. In cardiac diseases, xanthine oxidase (XO) activity is up-regulated. XO catalyzes the oxidation of xanthine to uric acid, generating superoxide. We hypothesized that enhanced XO activity may affect BH4 availability and function. **Methods:** To test whether BH4 and different pteridine derivatives have an impact on XO-mediated ROS-production, we evaluated inhibitory effects produced by incremental doses of BH4 and pteridines such as BH2, Bio, Pt and XPH2 on Xanthine/XO reaction, measuring the formation of urate as readout. **Results:** Urate formation from Xanthine/XO was inhibited by all pteridines tested, excepting for XPH2. Bio abolished the formation of urate at 20  $\mu$ M. Higher concentrations of Pt (100  $\mu$ M) were needed to achieve the same effect. BH4 and BH2 (200  $\mu$ M) partially inhibited Xanthine/XO-driven production of urate (approximately 50%). We demonstrated that the production of superoxide anion does not occur when BH4 reacts with XO. Therefore, we exposed increasing concentrations of BH4 to XO (0.1 U / ml) in phosphate buffer at pH 7 for 15 min at 37 ° C and analyzed the products by HPLC. We found that BH4 was converted to XPH2, without BH2 as an intermediate product. The latter was proved by Fenton reaction-derived hydroxyl radical-driven BH4 transformation. **Conclusions:** Our data show that increased XO activity irreversibly transforms BH4 to XPH2. This alteration may contribute to the onset and/or progression of endothelial dysfunction during the course of acute or chronic cardiac diseases.

##### RR5. NADH Accumulates During Early Ischemia in the Rat Heart as Detected by a Novel One-step Hplc Approach

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**Background:** Lack of oxygen due to myocardial ischemia alters the redox status of pyridine-dinucleotides. To directly test the latter possibility, we developed a method

to analyze NADH, NAD, NADPH, NADP levels in rat hearts subjected to ischemia-reperfusion injury. **Methods:** A reaction of the oxidized-nucleotides with cyanide in basic solution leads to two stable fluorescent products, allowing us to separate all four nucleotides (NADH, NAD, NADP, and NADPH) and to quantify them on one single chromatogram. Langendorff-perfused rat hearts underwent global ischemia (15, 30 and 60 min) followed by 30-min reperfusion. The heart-chloroform extracts were analyzed by HPLC. **Results:** The analysis of HPLC chromatogram series of nicotinamide-adenine-dinucleotides over different ischemia time points revealed a sustained increase in the NADH level. This rise was already visible 15 min after ischemia ( $667.51 \pm 51.07$  nmol./gr to 15 min. ischemia vs  $55.06 \pm 7.46$  nmol./gr control). When nicotinamide-adenine-dinucleotides phosphates are concerned, ischemia induced a significant NADPH decrease starting at 30 min of ischemia ( $69.55 \pm 3.25$  vs  $79.48 \pm 3.61$  nmol./gr tissue in controls,  $P = 0.0098$ ). These changes became even more evident after 60 min: NADPH levels dropped to  $30.17 \pm 3.59$  nmol./gr tissue ( $P = 0.00003$ ). **Conclusions:** Our study shows that during early ischemia NADH accumulates in the heart tissue, likely due to an anoxia-induced blockade of the Krebs cycle. When the no-flow condition was longer than 15 min, the capacity of generating NADPH impaired during late ischemia. Altered bioavailability of pyridine-dinucleotides may have repercussions on cellular function and viability during the I/R injury.

**RR6. Polyphenol Compounds in Sardinian Wines Can Modulate Redox Cell Signalling Induced by a Dietary Mixture of Oxysterols in Human Enterocyte-like CaCo-2 Cells**

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**Background:** The beneficial role of polyphenols in human health, acting mainly as direct antioxidants has been widely documented. These compounds are distributed in all foods of plant origin, and exert many biological activities. Polyphenol food concentration is extremely variable due to different seasonal and geographic variations of vegetable growth. Sardinian wines (Cannonau and Vermentino) are particularly rich in flavonoids and phenolic acids, which have long residence time and accumulate in the mucosal intestinal layer where they can exert their beneficial effects. In this study we report the role of Sardinian wine extracts in modulating the main redox signaling pathways activated in differentiated CaCo-2 cells by a dietary mixture of the most representative oxysterols found in cholesterol-rich foodstuff. **Methods:** Differentiated CaCo-2 cells, whose phenotype is similar to normal mucosa of small intestine, were treated with a mixture of oxysterols (7-ketocholesterol, 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol, 5 $\beta$ ,6 $\beta$ -epoxycholesterol, 7 $\alpha$ -hydroxycholesterol, and 7 $\beta$ -hydroxycholesterol). Cells were pre-treated with Cannonau or Vermentino extracts. The activation of colonic NADPH oxidase (NOX1) and of the main transduction molecules (JNK, ERK and p38MAPK) were evaluated by Western Blotting. Reactive oxygen species (ROS) generation was detected using 2',7'-dichlorofluorescein. **Results:** Pre-treatment with different wine extracts showed a decrease of ROS generation by decreasing NOX1 activity. Up-regulation of p38MAPK by dietary oxysterols was lowered by wine extracts. **Conclusions:** Our experimental model show that, besides their direct antioxidant action as free-radical scavengers, polyphenols present in Sardinian wines could exert their beneficial effects by interfering with red-ox cell signals, which involve NOX1 and p38MAPK down-modulation.

**SIGNAL TRANSDUCTION**

**ST1. Could Iron Overload Impair the Migratory Ability of Neurons? Evidence from a Cell-Based Model**

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**Background:** Iron is essential for proper brain development in the fetal and early neonatal period. Iron represents a micronutrient for cellular metabolism and aerobic respiration, but cellular iron overload produces toxic build-up in many organs (including brain) via free radical formation. In thalassaemic patients with pubertal failure, iron overload is the most important factor afflicting the hypothalamic-pituitary axis, leading to hypogonadotropic hypogonadism and growth failure. **Methods:** Mouse GN-11 cells (immature GnRH neurons with migratory ability) were used. Hcpidin, ferritin and transferrin receptor gene expression was evaluated by PCR. GN-11 chemotaxis was assessed by Boyden chamber assay. Activation of chemomigration-related cell signaling (extracellular signal-regulated kinase (ERK), 5' adenosine monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC)) was evaluated by Western blot analysis. **Results:** GN-11 cells express hcpidin, ferritin and transferrin receptor genes. 150  $\mu$ M ferric ammonium

citrate (FAC) treatment inhibited (-35%,  $P < 0.05$ ) FBS-induced chemo-migration of GN-11 cells, which was rescued by pre-treatment with 100  $\mu$ M deferoxamine, a specific iron chelator. Time-course experiments showed that 150  $\mu$ M FAC was able to phosphorylate both ERK and AMPK after 10 min treatment. Specific ERK and AMPK inhibitors, U0126 and Compound C, respectively, abolished FAC-mediated signaling. Moreover, U0126 and Compound C (both 10  $\mu$ M) counteracted FAC-driven phosphorylation of ACC, an AMPK downstream protein. **Conclusions:** The present data show that iron negatively affects neuron migration via ERK and AMPK. Among the consequences of this event, iron overload may impair migration of GnRH neurons from the olfactory placode into forebrain and hypothalamus, where they promote reproductive competence.

**ST2. PRDM Gene Products in Testicular Germ Cell Tumors**

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**Background:** Testicular germ cell tumors (TGCT) originate from primordial germ cells blocked at different stages during maturation, reflecting different histological tumor subtypes. A common genetic alteration in TGCT is a deletion of chromosome 1 short arm, where the *PRDM2* gene, a member of positive regulatory domain gene family, is located. Moreover recent studies demonstrated that members of PRDM gene family have an essential role in the early stages of testicular development. The aim of this study is to evaluate PRDM gene family members for a possible tumor-suppressor function in TGCT. **Methods:** PRDM gene expression was assessed by mRNA RT-PCR. Cells were treated with 100 nM 17 $\beta$ -Estradiol (E2), 100 nM DHT or 10  $\mu$ M RA in serum free medium for 24h. RNA interference was performed using BLOCK-iT™ Pol II miR RNAi system. Proliferation assay was performed with propidium iodide staining and FACS analysis. **Results:** In GC1 mouse spermatogonial cells treatment with proliferation agents 5 $\alpha$ -dihydrotestosterone (DHT) and E2 reduced PRDM2/RIZ1 expression levels whereas PRDM2 total forms showed no variation; the same treatment significantly increased PRDM4 and PRDM10 expression levels. Silencing *PRDM2* gene expression by RNA interference increased *PRDM10* expression levels and reduced the proliferation rate of spermatogonia. **Conclusions:** In spermatogonia as in MCF-7 cell line, E2 and DHT regulate *PRDM2* gene expression suggesting that *PRDM2* gene products could mediate the effect of these agents on cell cycle progression. *PRDM4* and *PRDM10* are also responsive to steroid hormones and *PRDM10* probably cooperates with *PRDM2*, as demonstrated by the increase of its expression levels after *PRDM2* gene silencing.

**ST3. NHERF1 and CFTR Overexpression Restore Tight Junction Organisation and Function in Cystic Fibrosis Airway Epithelial Cells Via the Involvement of Ezrin and the RhoA/ROCK Pathway**

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**Background:** The pathophysiology of cystic fibrosis (CF) lung disease is characterised by abnormal ion and fluid transport across the epithelium with neutrophil-dominated inflammatory response. Tight junctions (TJs) restrict the transit of molecules through the paracellular route and act as a barrier to regulate access of inflammatory cells into the airway lumen. In CF cells, NHERF1 overexpression rescues CFTR-dependent chloride secretion by inducing the formation of the multiprotein complex NHERF1-RhoA-ezrin-actin. In this context, we studied whether NHERF1 and CFTR are involved in the organisation and function of TJs. **Methods:** TJ organisation was studied by confocal microscopy on ZO-1, occludin, claudin-1 and JAM-1 in polarised wild-type (16HBE) and CF (CFBE) airway epithelial cells. Barrier function was studied by dextran permeability and neutrophil transmigration. **Results:** CFBE monolayers presented a disorganisation of TJ proteins as compared with 16HBE monolayers, paralleled by increased permeability to dextrans and neutrophil transmigration. Overexpression of NHERF1 or CFTR rescued TJ proteins to their proper location and restored the barrier function. Expression of a phospho-dead ezrin mutant, T567A, increased permeability in 16HBE and in a CFBE clone stably overexpressing NHERF1 (CFBE/sNHERF1), whereas a constitutively active form of ezrin, T567D, achieved the opposite effect in CFBE. A dominant-negative form of RhoA (RhoA-N19) disrupted ZO-1 localisation at the TJs and increased permeability in CFBE/sNHERF1. The inhibitor Y27632 of Rho kinase (ROCK) increased permeability as well. **Conclusions:** These data suggest a role for the multiprotein complex CFTR-NHERF1-ezrin-actin in maintaining TJ organisation and barrier function, and suggest that the RhoA/ROCK pathway is involved.

**ST4. The Akt1 Gain of Function Mutation, E17K, in Lung Epithelial Cells**

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**Background:** Aberrant signalling from the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is frequently observed in human cancer. Different studies have identified a gain-of-function mutation in the pleckstrin homology domain of Akt1 that results in a glutamic acid to lysine substitution at residue 17 (E17K) in multiple cancer types including lung carcinomas. So far the contribution of somatic Akt1 mutations to development of epithelial cancer has remained elusive. **Methods:** Herein, we examined the activity of the E17K mutant using immortalized human bronchial epithelial cells as model system (BEAS-2B cells). **Results:** Expression of Akt1-E17K mutant, but not of wild-type Akt1, in BEAS-2B cells induced multiple phenotypic alterations characteristic of tumour cells, including growth factor-independent DNA synthesis, anchorage-independent growth in soft agar, increased capability to migrate and invade, resistance to anoikis and tumorigenicity in nude mice. In addition, mutant Akt1 induced an expansion of a subset of putative tumor-initiating cells (TICs) as determined by an increase in the efficiency of sphere formation as well as by enhanced expression of stem cell markers, leading to the emergence of a cell population endowed with the capability to form aggressive, undifferentiated tumours at high efficiency (103-104 cells/injection). Knockdown of Oct-4 significantly inhibits the capability of BEAS-Akt1 cells to form sphere and to grow as xenograft *in vivo*. **Conclusions:** In summary, our data indicate that the Akt1-E17K mutant is oncogenic in lung epithelial cells and that the stem cell transcription factor Oct-4 is a key mediator of its oncogenic activity, thus contributing to the pathogenesis of lung cancer.

**ST5. Identification of Novel Estrogen Receptor- $\alpha$  (ER $\alpha$ ) Protein Interactors Reveals Significant Differences Among Antiestrogen Compounds in Human Breast Cancer Cells**

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**Background:** ER $\alpha$  is a ligand-activated transcription-factor that promotes mammary epithelial cell growth and breast-carcinogenesis controlling key cellular pathways via protein-protein interactions within co-regulator complexes. ER $\alpha$ -ligands are classified as agonists (estrogens:17 $\beta$ -estradiol/E2), mixed agonists-antagonists (SERMs:Tamoxifen/Tam and Raloxifene/Ral) and pure-antagonists (ICI182,780-Faslodex/ICI), according to response elicited in hormone-responsive cells.

Crystallographic analyses on ER $\alpha$  bound to E2 or anti-estrogens Tam, Ral and ICI revealed ligand-dependent receptor conformations leading to generation of specific binding-sites at protein surface that represent potential docking-sites for different co-regulators, still mainly unknown. Identifying ligand-specific ER $\alpha$ -interactors may lead to a better understanding of molecular mechanisms mediating anti-estrogen effects on breast cancer (BC). **Methods:** Tandem affinity purification (TAP) was applied to map nuclear ER $\alpha$ -interactomes induced by different ligands in hormone-responsive BC cells. For this purpose, MCF-7 cells stably expressing ER $\alpha$  fused to TAP-tag at the C-terminus were used to purify native receptor-containing nuclear multi-protein complexes from cells treated with either E2, SERMs or ICI. Isolated complexes were dissociated *in vitro* and analyzed by mass spectrometry (nanoLC-MS/MS). **Results:** This led to identification of a large number of novel ER partners and revealed significant differences among ligands tested. E2-promoted ER $\alpha$ -interactome (270 proteins) is different and more complex than those elicited by TAM (71), Ral (48) or ICI (54) that, in turn, are significantly different from each other. **Conclusions:** *In silico* analysis of molecular functions represented by these interactomes indicates that E2 induces receptor association with epigenetic, transcriptional and actin-polymerization factors. Moreover, although ICI-induced interactome is involved in negative regulation of biosynthetic processes, Tam- and Ral-dependent ones comprise regulators of macromolecular-complex synthesis and transcription-linked processes. Supported by AIRC; MUIR; Regione Campania; University of Salerno; Fondazione con il Sud; UICC-ICRETT fellowship to F. Cirillo; FEBS Short-Term Fellowships to G. Nassa.

**ST6. A Novel Morphogen-Dependent Control of IRES-Dependent Translation in Cancer and Development**

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**Background:** The Hedgehog morphogen is critical for development, stem/progenitor cell fate and is dysregulated in many cancers, such as medulloblastoma and basal cell carcinoma. Hedgehog signaling leads to the activation of Gli transcription factors and activation of gene expression, which is in part regulated by Suppressor of Fused (Sufu). To date it is not known whether Hedgehog also regulates protein translation. **Methods:** We have used biochemical, cell and stem cell culture techniques, as well as *in vivo* gene knockdown or overexpression studies in *Drosophila melanogaster*. **Results:** We show here that Hedgehog directly regulates IRES-dependent translation via a protein complex of Sufu and the RNA-binding protein CNBP. CNBP is up-regulated by Hedgehog and then, in a complex with Sufu, is recruited to 5'UTR sequences of target mRNAs where it promotes IRES-dependent protein translation. Sufu protects CNBP from ubiquitination and proteasomal degradation, thus promoting its activity. Consistent with the developmental and tumorigenic role of Hedgehog, CNBP is up-regulated in cerebellar stem cells, medulloblastomas and tumor stem cells, where it mediates self-renewal and cancer growth. Furthermore, this mechanism is also conserved in *Drosophila*, where CNBP directs wing development in synergy with Ci, the *Drosophila* Gli homolog. **Conclusions:** Taken together, we demonstrate that Hedgehog-dependent physiologic and tumorigenic processes require a conserved IRES-dependent translational control. We propose that the translational mediator CNBP and the IRES-dependent pathway can be considered promising novel targets for anti-cancer approaches.

**ST7. Itch-Dependent Nondegradative Ubiquitination of SuFu Controls Hedgehog Pathway**

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**Background:** Hedgehog (Hh) pathway regulates tissue patterning and cell proliferation through the activation of the transcription factors belonging to the Gli family. Gli proteins display a positive function, but can also be converted into a cleaved form with transcriptional repressor activity. This process is finely tuned by SuFu, which in the absence of signaling interacts and protects Gli3 full-length from degradation and promotes its conversion into a repressor form, Gli3R. The mechanistic aspects of this process are however poorly understood. **Methods:** Mouse fibroblasts (NIH3T3) and human epithelial kidney (HEK293) cells were used in this study. Protein-protein interaction was assessed by immunoprecipitation and GST-pulldown, ubiquitin modification by ubiquitination *in vivo* and *in vitro* assays. Depletion of Itch was obtained by siRNA. **Results:** We show here that the conversion of Gli3 into a repressor form is regulated by Itch, an HECT E3 ubiquitin ligase involved on major biological processes. Itch binds SuFu and promotes its ubiquitination both *in vivo* and *in vitro*. Of relevance, this Itch-mediated ubiquitination is insensitive to the proteasome activity and does not affect SuFu stability, suggesting that such a process drives a regulatory rather than a proteolytic response. Indeed, Sufu ubiquitination increases the binding of Gli3 to Sufu, thereby leading to Gli3R formation and inhibition of the Hh pathway. Of note, Hh agonists prevent Itch-triggered ubiquitination of SuFu, thus explaining how this process is regulated by the signaling pathway. **Conclusions:** Our findings suggest that Itch-dependent SuFu ubiquitination, regulating dynamic protein-protein interaction, plays an important role in the control of Hh signaling.

**STEM CELLS IN TISSUE REGENERATION AND REGENERATIVE MEDICINE**

**SC1. Human Amniotic Mesenchymal Stem Cells Modify the Function and Cytokine Production of F508del Airway Epithelial Cells Upon Coculture**

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**Background:** In cystic fibrosis (CF), there is a lack/dysfunction of CF transmembrane conductance regulator (CFTR), a chloride channel, and epithelial sodium channel (ENaC) at the level of the respiratory epithelium. We evaluated human amniotic mesenchymal stem cells (hAMSCs) obtained from end-term placentae for their ability to modify defective function of CF respiratory epithelial cells. **Methods:** hAMSCs and CF bronchial epithelial cells (CFBE41o-) were cocultured on Transwell filters seeded at different ratios. Real-time PCR was used to study transcript levels of CFTR and ENaC. Expression of the CFTR protein was

investigated by flow cytometry and confocal microscopy. Chloride efflux was studied by fluorimetry. ENaC activity was assayed by apical fluid reabsorption. Cytokine secretion was studied in the apical and basolateral conditioned media. **Results:** hAMSCs expressed at low levels CFTR mRNA and gamma, but not alpha and beta, subunits of ENaC. Cocultures of hAMSCs with CFBE cells demonstrated that at least 50-80% of hAMSCs acquired a detectable CFTR expression on the apical membrane above the background. Fluorimetric measure of ion chloride efflux allowed to detect an increased function of the CFTR channel in cocultures as compared with CFBE cells and hAMSCs alone. Amiloride-dependent fluid reabsorption decreased when CFBE cells were cocultured with hAMSCs compared to CFBE41o- cells alone. Unexpectedly, IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  showed an increase depending on the hAMSC-CFBE ratio. **Conclusions:** Overall, these data show that hAMSCs are capable of resuming some pathological features of CF airway epithelial cells, although the cellular and molecular mechanisms have to be deciphered.

### SC2. Stem-Like Cells in Nephrospheres Present Multilineage Differentiative Abilities

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**Background:** To isolate stem cells from adult kidney, we used the sphere forming assay approach coupled with the evaluation of self renewal and differentiative abilities. **Methods:** Nephrospheres were obtained culturing the cells from adult kidney in specific conditions. The sphere forming efficiency (SFE) was calculated. To identify and isolate the stem cell population within the nephrospheres the PKH dye, retained in quiescent cells, was used. The ability of nephrosphere forming cells and stem cells to differentiate into epithelial, podocytic and endothelial lineages was evaluated using specific media and 3D cultures performed with semisolid substrates. The regenerative abilities were evaluated transplanting the cells under the renal capsule of nude mice. **Results:** After 12 days in culture, we obtained nephrospheres propagable for at least eleven passages, with a SFE of 0.8%. The spheres are composed of a heterogeneous population of stem cells, a more PKH fluorescent (PKHhigh) population, and progenitors that are less fluorescent (PKHlow/neg). Nephrosphere forming cells and PKHhigh cells can differentiate into epithelial, podocytic and endothelial lineage; they can form tridimensional hollow structures miming the tubular *in vivo* behavior. Nephrosphere forming cells can generate tubular like structure *in vivo*, whereas PKHhigh cells inserted into the mice parenchyma maintain their undifferentiated status. **Conclusions:** Nephrospheres contain stem-like cells, as shown by the presence of quiescent cells able to self renew and by the ability to differentiate into renal multilineage phenotypes. This PKHhigh stem cells can be isolated and characterized and can represent a cellular material USble in regenerative medicine.

### SC3. Influence of Fibronectin and Collagen as Substrates for the Isolation and Expansion of Endothelial Progenitor Cells from Human Peripheral Blood

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**Background:** Endothelial progenitor cells (EPCs) have a crucial role in endothelial homeostasis. Among populations of endothelial cells that can be cultured from adult blood, endothelial colony-forming cells (ECFCs) are considered the true EPCs. Different methods can be used to isolate and expand ECFCs from peripheral blood, the main difference consisting in the substrate (fibronectin or collagen) used for cell seeding. The possible impact of the substrates on ECFC cultures has not been investigated, so far. In this study we compared ECFCs obtained using fibronectin or collagen during ECFC isolation and/or expansion. **Methods:** Healthy donor PBMCs were seeded on fibronectin or collagen and cultured in EGM-2. ECFC colonies were released from the original tissue-culture plates and replated onto tissue-culture flasks precoated with fibronectin or collagen, for further passages. All cultures were analyzed for isolation of ECFC colonies, cell yield after serial passaging, immunophenotype, cytokine production and *in vitro* angiogenesis. **Results:** Fibronectin sustained ECFC isolation more efficiently than collagen because, although similar numbers of colonies were obtained on the two substrates, ECFC colonies appeared earlier on fibronectin ( $P < 0.05$ ). Collagen sustained ECFC expansion more efficiently, as ECFCs expanded on collagen showed longer survival ( $P < 0.02$ ), lower rate of cultures undergoing senescence at early passages ( $P < 0.02$ ), and a higher cell yield. Preliminary results indicate that ECFCs expanded on

fibronectin or collagen have similar immunophenotypes, cytokine profiles and ability for *in vitro* tubulogenesis. **Conclusions:** We suggest that isolation on fibronectin, followed by expansion on collagen, may represent the most efficient strategy to obtain ECFCs from peripheral blood samples.

### SC4. Mobilisation of Hematopoietic Stem/Progenitor Cells in Acute Lung Injury: Role of VLA-4

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**Background:** The aim of this study was to determine whether there is a relationship between pulmonary inflammation, expression of VLA-4 (CD49d), LFA-1 (CD11a), and L-selectin (CD62L) and chemotaxis in resident hematopoietic stem/progenitor cells (HSPCs), as well as on their mobilisation in the blood. **Methods:** At 24, 48 and 72 h following an intratracheal administration of a single LPS bolus in C57Bl/6 mice, pulmonary inflammation was studied in cytopins and bronchoalveolar lavage fluid (BALF) specimens. Expression of CD49d, CD11a and CD62L was analysed in Sca-1+ HSPCs and subpopulations as well as in circulating Sca-1+ blood cells by flow cytometry. SDF-1-directed transmigration through an endothelial cell sheet was investigated. **Results:** In coincidence with a peak of neutrophils, cytokine (IL-1, TNF- $\alpha$ , IL-6) and chemokine (KC, MIP-2, SDF-1) levels in BALF at 48 h, the number of marrow HSPCs expressing CD49d increased. The number of CD49d-positive HSPCs dropped at 72 h. The HSPC subset comprising bigger cells behaved the same. A significant decrease of circulating Sca-1+ cells in the mononuclear population, but not in the polymorphonuclear granulocytes, at 72 h following LPS administration was observed. Finally, SDF-1 directed chemotaxis of marrow HSPCs subset of bigger cells was higher in cells obtained from LPS-treated animals than those from controls at 72 h. **Conclusions:** Our data provide evidence for a temporal relationship between CD49d level fluctuation in HSPCs, their mobilization from the bone marrow and decrease in circulating HSPCs, likely for their influx in the inflamed lung, and show that the HSPC bigger subpopulation is affected by these changes.

### SC5. Erk1/2-Oct4A Interaction Mediates Oct4A Phosphorylation and Degradation

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**Background:** Embryonic stem (ES) cell self-renewal properties are attributed to critical Oct4A amounts. Although the Oct4A transcriptional targets have been deeply studied, little is known about its post-translational regulation. Sequence analysis revealed that Oct4A contains five putative ERK1/2 phosphorylation sites. **Methods:** We were able to show that Oct4A interacts with ERK1/2 in Ntera2 cell line, using both *in vitro* GST-pull down and *in vivo* co-immunoprecipitation assays. To explore the mechanism of ERK1/2-Oct4A interaction, we performed mass spectrometry analysis on HeLa cells transfected with Oct4A and MEK1CA. To investigate the possibility that ERK1/2 activation can enhance Oct4A degradation, we analyzed endogenous ubiquitination in HeLa cells transfected with Flag-Oct4A alone or with MEK1CA. **Results:** Consistent with the hypothesis that Oct4A is a putative ERK1/2 substrate, we were able to show that Oct4A interacts with ERK1/2 in Ntera2 cell line, using both *in vitro* GST-pull down and *in vivo* co-immunoprecipitation assays. To explore the mechanism of ERK1/2-Oct4A interaction, we performed mass spectrometry analysis on HeLa cells transfected with Oct4A and MEK1CA and we identified phosphorylation of Ser 111, one of the previously evidenced phosphorylation sites. When we examined ubiquitination of Oct4A from the FLAG immunoprecipitation, we saw that the extent of Oct4 ubiquitination was clearly increased when MEK1CA was co-expressed and this increase was more evident after MG132 treatment, a proteasomal inhibitor. **Conclusions:** These results suggest an increase in Oct4A ubiquitination downstream of MEK1 activation. Understanding and controlling this mechanism by which stem cells balance self-renewal would substantially advance our knowledge of stem cells and their clinical application.

### SC6. Proteomic Profile of CD24+ CD133+ Renal Multipotent Progenitors (RMP)

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**Background:** RMP represent a population of undifferentiated pluripotent cells with both self-renewal and multilineage-differentiation characteristics. A population of CD24+CD133+RMP in adult human kidneys is able to repair injured renal tissue.

Proteomics provides a powerful approach for studying the characteristics of RMP and discovering molecular markers. **Methods:** RMP lines were isolated from normal kidneys of 30 patients undergoing nephrectomy for renal cell carcinoma. We have analyzed proteome profiles of two RMP lines using 2-DE analysis combined to nano-HPLC-ESI-ion trap and MALDI-TOF-MS analysis. The identified proteins were studied by ingenuity pathway analysis (IPA) and validated by Immunoblot analysis.

**Results:** An average of about 1080 spots were detected in the silver stained gels of total protein extract. The protein spots identified were involved in cellular cytoskeleton (28.6%), stress-response (23.8%), cellular metabolism (14.3%), cell-proliferation and differentiation (9.5%). A large number of proteins were identified as chaperones, heat-shock-proteins, ubiquitin/proteasome, and oxidative-stress-responsive-proteins. Functional clustering of differentially expressed proteins by IPA TM in comparison with the proximal tubular epithelial cell proteome from the same donors revealed that 17 $\beta$ -estradiol-pathway was overexpressed in RMP (IPA score=32). To confirm this observation we investigated the expression of three up-regulated key-proteins of the pathway (17 $\beta$ -estradiol receptor, NME1, Zyxin) by immunoblot analysis and observed a significant increase of their expression in RMP cell lines compared to PTEC from the three donors. **Conclusions:** This study represents the first proteomic dataset for RMP and may provide a better insight into RMP-biology. Several studies explore the direct effects of sex-hormones on kidney and our data may suggest that RMP may represent a key-target for 17 $\beta$ -estradiol. Knowledge of RMP-biology may enable a better comprehension of the mechanisms of renal repair.

**SC7. Pharmacologic Attenuation of Cardiac Stem Cell Senescence**

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**Background:** We demonstrated that both age and pathology exert detrimental effects on human cardiac stem cells (CSC) and are associated with reduced telomerase activity and telomere length, telomere erosion, telomere induced dysfunction foci and CSC dysfunction *in vitro*. Our aims were to investigate whether CSC senescence is associated with their reduced reparative ability *in vivo*, to identify the molecular determinants possibly responsible for CSC senescence, to screen drugs (i.e. rapamycin, resveratrol, and DETA/NO) able to interfere with CSC senescence, and to verify if CSC drug treatment *in vitro* is effective in restoring the reparative potential of senescent CSC *in vivo*. **Methods:** CSCs were isolated both from normal (D) and failing (F) human hearts. The reparative capacity of CSC was evaluated in a SCID/beige mouse model of acute myocardial infarction (AMI). Echocardiography and cardiac catheterization were performed 2 weeks post-AMI. Fibrosis, angiogenesis, myocyte growth, myocyte apoptosis, and myocyte senescence were assessed. Western blot analysis of CSC was performed to identify pathways possibly associated with CSC senescence. Drug-screening assays were performed treating F-CSC for three days and analyzing them in terms of cell senescence, proliferation, and death. **Results:** Pathology attenuates the reparative ability of CSC *in vivo*. The short-term pharmacological treatment of CSC resulted in a significant reduction in p16+, p21+, and  $\gamma$ H2A.X+Ki67- senescent cells, together with an increase in CSC proliferation. Last, *in vitro* treatment of F-CSC with 10nM rapamycin and 0.5 $\mu$ M resveratrol prior to their *in vivo* administration to infarcted mice restored their reparative ability *in vivo*, significantly improving global heart function. **Conclusions:** Pharmacological inhibition of CSC senescence enhances their regenerative capacities.