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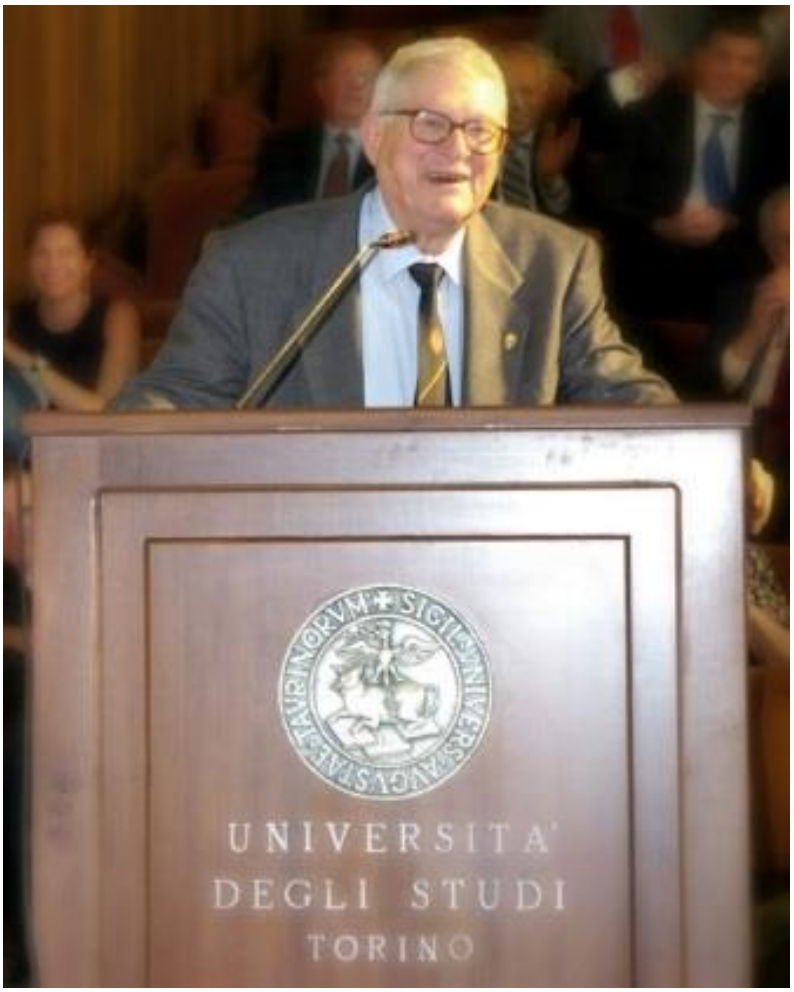
ORAL

COMMUNICATIONS

Session I

Oxidative Stress

Honoring Prof. Mario Umberto Dianzani



Controversial role of oxysterols in the Alzheimer's disease pathogenesis

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by amyloid plaques and neurofibrillary tangles of hyperphosphorylated tau protein. A growing amount of evidence suggests a link between altered lipid metabolism in the brain and AD development; moreover, AD is tightly related to neuroinflammation and oxidative stress. To prevent its accumulation, brain cholesterol is converted into 24-hydroxycholesterol (24-OH) which, unlike cholesterol, can cross the blood brain barrier (BBB). Another oxysterol, 27-hydroxycholesterol (27-OH), is produced in situ in the brain, however, most 27-OH flows from the circulation into the brain, since it can cross the BBB. 24-OH and 27-OH play a fundamental role in AD development causing neurodegeneration but 24-OH may also exert neuroprotective effects.

We investigated the possible role of 24-OH and 27-OH in the pathogenesis of AD. A preliminary study was carried out on autopsy samples of frontal cortex from human AD (early and late AD) and normal brains: 24-hydroxylase expression and the levels of its product 24-OH were significantly decreased in late AD; conversely, 27-hydroxylase expression and the levels of its product 27-OH were significantly increased with the severity of the disease. In late AD cases we also observed the upregulation of some proinflammatory molecules (IL-8 and MMP-9) compared to control cases. In accordance with these observations, our i*n vitro* studies showed that both 24-OH and 27-OH induce the expression of CD36, β_1-integrin, IL-8, MCP-1 and MMP-9 in SH-SY5Y cells, via TLR-4/COX-2/mPGES-1. We also investigated the possible effect of 24-OH and 27-OH on neurotoxic phospho-tau accumulation in AD by modulating the neuroprotective SIRT-1 pathway in SK-N-BE cells. Although both oxysterols induced the synthesis of the neurotoxic amyloid $\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$), surprisingly, 24-OH reduced tau phosphorylation, while 27-OH stimulated the latter process. This peculiar behavior of 24-OH depends on its enhancement of ROS generation and the consequent SIRT-1 upregulation, which leads to PGC-1α deacetylation, GSK-3β inactivation and, at the end, to the beneficial tau phosphorylation decrease.

These results clearly support the involvement of oxysterol 24-OH and 27-OH in neuroinflammation and Aβ deposition; concerning phospho-tau accumulation, only 27-OH contributes to tau pathology, while 24-OH exerts a protective effect.

HO-1-derived bilirubin protects endothelial cells against hyperglycemia

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Hyperglycemia is associated with endothelial cell dysfunction leading to the progression of diabetic vascular pathology. The generation of oxidative stress is recognized as the main factor in pro-inflammatory endothelial cell activation or damage in diabetes. However, cells have evolved highly regulated defense systems to counteract the overproduction of reactive oxygen species (ROS). As a matter of fact, the transcription factor Nuclear factor erythroid 2-related factor (Nrf2) is activated in response to ROS generation and up-regulates a plethora of antioxidant genes. Among these, heme oxygenase 1 (HO-1) has been described to exert a strong protective role against oxidative stress, through the antioxidant and antiapoptotic properties of its metabolic products bilirubin, derived from biliverdin, ferritin, induced by free iron and carbon monoxide. We showed that the exposure of Bovine Aortic Endothelial Cells (BAEC) to 25mM Glucose (25G) up-regulated the binding of Nrf2 to the Antioxidant Responsive Element (ARE) DNA sequences and increased HO-1 protein expression, preventing cell death. HO-1 inhibition strongly decreased cell viability increasing ROS generation and the further addition of bilirubin prevented cell death. Moreover, we showed that bilirubin exerted cell protection reducing the amount of HNE derived from cells exposed to 25G and treated with HO-1 inhibitor. In conclusion, we demonstrate that endothelial cell resistance against glucose-induced damage depends on the production of bilirubin from HO-1, emphasizing the importance of the activation of Nrf2/HO-1 pathway in the induction of an hormetic response in endothelial cells during hyperglycemia.

Grants from Genoa University

Modulation of glutathione levels in neuroblastoma cell line: key role in multi-drug resistance

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Neuroblastoma (NB), a very common paediatric tumor, is initially sensitive to treatment, but subsequently, becomes chemoresistant by enhancing its antioxidant defence. In fact, among the adaptive mechanisms, the increase in the levels of glutathione (GSH), one of the most important antioxidant molecules, plays a key role.
In order to investigate the redox-modulated mechanisms responsible for chemoresistance, an etoposide-resistant cell line (Etopo-R) was selected by treating HTLA-230, that is high-risk NB cells, for 6 months with increasing doses of etoposide, a clinically-used drug, and then studied.
Cell viability analysis shows that Etopo-R cells were more resistant to high concentrations of etoposide, doxorubicin and H_2O_2 than parental cells and that they did not undergo DNA damage after etoposide treatment whereas clonogenic assay shows that Etopo-R were more tumorigenic than HTLA after etoposide treatment. Moreover, fluorescence analysis highlights that GSH levels of Etopo-R, in comparison to HTLA, were increased by 70% and PCR analysis shows an overexpression of γ-glutamyl-cysteynil ligase, a crucial enzyme in GSH biosynthesis. Furthermore, cytofluorimetric analysis shows that Etopo-R cells produced a lower amount of ROS in respect to HTLA when treated with scalar doses of etoposide and doxorubicin. In fact, in both cell lines, co-treatment of etoposide with butionine sulfoximine (BSO, a GSH depleting agent) was able to increase ROS levels and to enhance the anti-tumorigenic effect of the drug while the co-treatment with N-acetil-cysteine (NAC, a promoter GSH biosynthesis) prevented etoposide-induced ROS production and counteracted the anti-tumorigenic effect of etoposide.
Collectively, our results suggest that the major content of GSH in Etopo-R cells, by reducing ROS levels, is crucially involved in the development of Multi-Drug Resistance and malignant phenotypes underlining the importance of GSH as a therapeutic and predictive target.

Grants from Genoa University

Fat-laden cells release microparticles that activate NLRP3 inflammasome in HepG2 cells and macrophages

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Fat-laden liver cells undergoing lipotoxicity can release pro-angiogenic micro-particles (MPs) that may have a role in the pathogenesis of NAFLD/NASH. Here we investigated whether MPs released by fat-laden cells may affect in a paracrine way NLRP3 inflammasome (known to be activated in vivo in NAFLD/NASH). MPs released from fat-laden HepG2 (i.e., HepG2 exposed for 24 hr to 0.25 mM palmitic acid or PA) were added to HepG2 resting cells or to activated human THP1 macrophages. Expression of NLRP-3, pro- and cleaved caspase1, pro- and cleaved IL-1β was evaluated by Western blot, ELISA assays or qRT-PCR. MPs were rapidly internalized by both cell types, as revealed by means of confocal microscopy. MPs induced a time-dependent increase in the expression of NLRP3 inflammasome components in resting HepG2 cells starting from 6 hrs and then reaching a plateau at 16-24 hrs, with a kinetics overlapping the one exerted by PA and delayed as compared to LPS (1-3 hrs). MPs and PA, but not LPS, induced caspase-1 activation and consequent release of IL-1β in a time-dependent manner. MPs also up-regulated NLRP3 inflammasome expression in THP1 human macrophages within 3-6 hrs, resulting in a significantly increased release of IL-1β. Fat-laden cells, by releasing MPs in a paracrine way, can efficiently trigger inflammasome activation in surrounding hepatic cells as well as macrophages, thus identifying an additional new molecular mechanism of inflammation in NASH pathogenesis.

CX_3CR1-expressing inflammatory dendritic cells are involved in the progression of chronic liver injury

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In healthy livers, dendritic cells (DCs) represent a small fraction of non-parenchymal cells and have a predominant tolerogenic phenotype, but a dramatic DC expansion occurs in chronic liver disease in combination with a stimulation in their antigen presenting activity and the release of pro-inflammatory cytokines. Liver monocytes play a major role in the development of chronic liver injury. In inflamed tissues, monocytes can differentiate in both macrophages and DCs. In this study, we have investigated the role of monocyte-derived inflammatory dendritic cells (IDCs) in experimental model of chronic steatohepatitis induced by feeding mice with a methionine-choline deficient (MCD) diet.

The progression of experimental steatohepatitis was associated with an expansion of myeloid DCs featuring the combination of inflammatory monocyte markers (F4-80, Ly6C) and the fractalkine receptor (CX_3CR1). These CX_3CR1^+ cells were also characterized by a sustained TNF-α production, suggesting monocyte differentiation to IDCs. The accumulation of CX_3CR1^+ IDCs in advanced NASH paralleled with the lowering in hepatic phasmocytoid and lymphocytoid DCs and was associated with an elevation in hepatic and circulating TNF-α levels and the worsening of parenchymal injury. Hydrogen sulfide (H_2S) has been shown to interfere with CX_3CR1 up-regulation in monocyte-derived cells exposed to pro-inflammatory stimuli. Treating 4 weeks MCD-fed mice with the H_2S donor NaHS while continuing on the same diet prevented the accumulation of TNF-α-producing CX_3CR1^+-IDCs without interfering with hepatic macrophage functions. Furthermore, NaHS reduced hepatic and circulating TNF-α levels and ameliorated transaminase release and parenchymal injury.

Altogether, these results indicate that inflammatory CX_3CR1^+-IDCs contribute in sustaining lobular inflammation during the progression of chronic liver injury.

Session II

Oncology I

The Extracellular signal-Regulated Kinase 5 is required for human melanoma cell growth

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Melanoma is the most aggressive and lethal among skin cancers, known for its high metastatic potential, enhanced heterogeneity, and resistance to chemotherapy. Mitogen-Activated Protein Kinases are often targets for the treatment of cancer, including melanoma. Nevertheless, available treatments for melanoma, especially in its intermediate or advanced stages, are unsatisfactory. The possibility of an involvement of the MAPK Extracellular signal-Regulated Kinase 5 (ERK5) in the growth of melanoma is an unexplored issue.

Cell lines and patient-derived primary melanoma cells (SSM2c, M32 and M26c, expressing wild type B-RAF, A375, M32 and SKMEL5 cells expressing V600E mutated B-RAF) have been used for in vitro experiments and xenografts in mice. ERK5 inhibition was obtained using XMD8-92, an ERK5 inhibitor, or BIX02189, a MEK5 inhibitor, or lentiviral vectors encoding for ERK5 specific shRNA.

In silico data analysis indicated that components of the ERK5 pathway may be altered in up to 40% melanoma patients. We found that ERK5 protein is consistently expressed in melanocytes, A375 melanoma cell line and in primary melanoma cells derived from several patients. Pharmacologic ERK5 inhibition dose-dependently decreased melanoma cell number in culture in either cells expressing wt (IC50 after 72 hours 3-3.5 μM) or V600E B-RAF (IC50 after 72 hours 2.1-2.5 μM). Consistently, genetic inhibition of ERK5 markedly decrease the number of cells in culture in patient derived melanoma cells expressing wt or V600E mutated B-RAF. Treatment with XMD8-92 induced G2/M arrest of wt B-RAF expressing cells and an increase of the percentage of cells in S phase, at the expense of those in G0/G1 in cells expressing V600E B-RAF. Genetic inhibition of ERK5 in SSM2c and A375 impaired melanoma cell growth in xenografts in mice. Combination of XMD8-92 or BIX02189 with vemurafenib determined additive inhibitory effect on colony formation of cells expressing V600E B-RAF. ERK5/BMK1 may be a suitable target for the treatment of melanoma. The recent development of ERK5 inhibitors allows translating experimental findings to a preclinical setting.

Newly identified mutation in the transcription factor GTF2I as a driver event in Thymic Epithelial Tumors

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Mutations of proteins involved in signal transduction are frequent in cancer. GTF2I controls transcription in response to growth factors including those that activate MAP kinase pathway. We performed whole exome sequencing of normal and tumor DNA from 28 thymic epithelial tumors (TETs) using HiSeq2000 or GA-II (Illumina). A single nucleotide mutation of GTF2I was observed in 6 thymomas (chr7:74146970T/A, according to the NCBI 37 version of the human genome). The mutation was missense (leucine to histidine), not previously described in cancer or as a polymorphism in dbSNP137 database. The mutation was predicted to alter the structure of the protein or its function according to Poliphen2 and SIFT algorithms. GTF2I mutation was present in 57% of TETs of an independent cohort of 268 formalin fixed paraffin embedded samples, being more common in A (82%) and AB (74%) thymomas. Patients with GTF2I mutation had a better disease related survival than those without (96% vs 70% 10-year survival, respectively; Log-Rank $p<0.001$). TETs predominantly express two splicing variants of GTF2I: β and δ isoforms. These variants with or without the identified mutation were ectopically expressed in NIH-3T3 cells using the plenti6.3/V5-Dest vector (Invitrogen). NIH-3T3 cells transfected with GTF2I grow faster in CellTiter 96 AQ assay (Promega) than mock controls. Mutated β and δ isoforms enhance cell proliferation more than the respective wild types. In soft agar, there were not statistically significant differences in colony formation between wild type and mutated isoforms. The mutant clones (both β- and δ-isoforms) exhibited higher levels of GTF2I protein. Using cycloheximide, protein synthesis was inhibited and a slower degradation of mutant compared to wild type GTF2I was observed. In conclusion, GTF2I mutation is frequent in thymic epithelial tumors, especially in A-AB histotypes and promotes proliferation of tumor cells probably as a consequence of a more stable form of the protein.

HDAC2 deregulation in tumorigenesis is causally connected to repression of immune modulation and defense escape

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Histone deacetylase 2 (HDAC2) is overexpressed or mutated in several disorders such as hematological cancers, and plays a critical role in transcriptional regulation, cell cycle progression and developmental processes. Here, we performed comparative transcriptome analyses in acute myeloid leukemia to investigate the biological implications of HDAC2 silencing versus its enzymatic inhibition using epigenetic-based drug(s). By gene expression analysis of HDAC2-silenced vs wild-type cells, we found that HDAC2 has a specific role in leukemogenesis. Gene expression profiling of U937 cell line with or without treatment of the well-known HDAC inhibitor vorinostat (SAHA) identifies and characterizes several gene clusters where inhibition of HDAC2 'mimics' its silencing, as well as those where HDAC2 is selectively and exclusively regulated by HDAC2 protein expression levels. These findings may represent an important tool for better understanding the mechanisms underpinning immune regulation, particularly in the study of major histocompatibility complex class II genes.

YAP activation protects urothelial cell carcinoma from treatment-induced DNA damage

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Current standard of care for muscle-invasive urothelial cell carcinoma (UCC) is surgery along with perioperative platinum-based chemotherapy. UCC is sensitive to cisplatin-based regimens, but acquired resistance eventually occurs, and a subset of tumors is intrinsically resistant. Thus, there is an unmet need for new therapeutic approaches to target chemotherapy-resistant UCC. Yes-associated protein (YAP) is a transcriptional co-activator that has been associated with bladder cancer progression and cisplatin resistance in ovarian cancer. In contrast, YAP has been shown to induce DNA damage associated apoptosis in non-small cell lung carcinoma. However, no data have been reported on the YAP role in UCC chemo-resistance. Thus, we have investigated the potential dichotomous role of YAP in UCC response to chemotherapy utilizing two patient-derived xenograft models recently established. Constitutive expression and activation of YAP inversely correlated with in vitro and in vivo cisplatin sensitivity. YAP overexpression protected while YAP knockdown sensitized UCC cells to chemotherapy and radiation effects via increased accumulation of DNA damage and apoptosis. Furthermore, pharmacological YAP inhibition with verteporfin inhibited tumor cell proliferation and restored sensitivity to cisplatin. In addition, nuclear YAP expression was associated with poor outcome in UCC patients who received perioperative chemotherapy. In conclusion, these results suggest that YAP activation exerts a protective role and represents a pharmacological target to enhance the anti-tumor effects of DNA damaging modalities in the treatment of UCC.

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Role of YAP signalling deregulation in the genetic susceptibility to hepatocarcinogenesis, and stemness and aggressivity of liver cancer

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Background and aims. YAP upregulation was investigated during hepatocellular carcinoma progression (HCC). We analyzed the connection of YAP deregulation with genetic susceptibility to chemically induced hepatocarcinogenesis and HCC stemness and aggressivity.
Methods. Experimental HCCs were induced in F344 and BN rats, genetically susceptible and resistant to hepatocarcinogenesis, respectively. Human HCCs with poorer prognosis (< 3 years survival, after partial liver resection, HCCP) and HCCs with better outcome (> 3 years survival; HCCB) were archival samples. Gene expression was evaluated by quantitative PCR and immunoblotting, and functional experiments were done with HepG2, Huh7, and Hep3B liver cell lines.
Results. Higher upregulation of Yap and of its target Ctgf, in F344 rat HCC than in BN HCC, was associated with highest increase in Yap-tyr357, p73 phosphorylation, and Caspase 3 cleavage in BN HCC. Upregulation of YAP, CTGF, 14-3-3 and YAP-14-3-3 complex, TEAD and YAP-TEAD complex in HCC reached highest values in HCCP. In contrast, YAP-ser127 decreased in HCC showing lowest values in HCCP, and YAP-tyr357, p73 phosphorylation and Caspase 3 cleavage showed highest increase in HCCB. Stem cell markers NANOG, OCT3-4, and CD133 progressively increased from HCCB to HCCP and was significantly correlated to YAP and YAP-TEAD expression. Growth rate was 1.5-2 times higher, between 48 and 96 hours, in HepG2, Huh7 and Hep3B cells transfected with pYap. This was associated, at 48 hours, with higher YAP and NANOG, OCT3-4, and CD133 expression in all cell lines used. YAP downregulation by specific siRNA in Huh7 and HepG3 cells led to significant decrease in NANOG, OCT3-4, and CD133 expression.
Conclusions. Our results indicate: (1) YAP signalling deregulation in HCC is under genetic control. (2) Genetic resistance to HCC is associated with highest phosphorylation of Yap at tyr357 and p73, and highest apoptosis. (3) YAP changes, favoring the formation of YAP-14-3-3 and YAP-TEAD complexes, associated with cell survival and upregulation of stem cell markers, contribute to HCC aggressiveness. In contrast, YAP changes favoring apoptosis, such as phosphorylation at tyr357, are associated with better HCC prognosis. (4) YAP upregulation favors HCC stemness and aggressiveness.

Session III

Oncology II

Prostate Specific Membrane Antigen commandeers prostate cancer signal transduction pathways to promote prostate cancer progression

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Prostate Specific Membrane Antigen (PSMA) expression is highly induced on tumor vasculature and on epithelial cells of advanced metastatic prostate cancer (PCa) where its expression correlates negatively with prognosis. While this upregulation suggests that PSMA may contribute to PCa progression and metastasis, functional confirmation remains elusive, thus prompting the current study. We crossed PSMA-null mice with TRAMP PCa transgenic animals resulting in PSMA-positive or negative tumors. Overall, PSMA-null tumors were smaller and of lower-grade with fewer blood vessels, consistent with PSMA regulating angiogenesis. PSMA-null tumors also showed increased levels of apoptosis. Additionally, wild-type tumors expressed high levels of IGF1R and survival proteins in the AKT/PI3K pathway, while loss of PSMA not only decreased expression of IGF1R by more than 50%, its deletion also diverted signaling to the MAPK/ERK1/2 pathway. Moreover, mouse and human PCa cells where PSMA levels had been altered recapitulated this signaling pathway switch. Finally, analysis of tumor mRNA microarray data from 31 human prostate adenocarcinomas and matched normal controls (cBioPortal) revealed that expression level of proliferation and anti-apoptotic markers Ki67 and survivin, as well as IGF1R positively correlate with PSMA levels in PCa patient specimens, thus supporting the translational importance of PSMA in PCa disease and progression. Taken together, our results suggest that an increase in PSMA within the prostate tumor epithelium effectively shifts cell signaling from an active GRB2-ERK1/2 pathway – inactive AKT/PI3K pathway state to an active AKT/PI3K pathway – inactive GRB2-ERK1/2 pathway state resulting in a pro-tumorigenic, anti-apoptotic phenotype.

The role of androgen receptor in prostate cancer-associated fibroblasts

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The androgen receptor (AR) controls transformation of prostate tissue and is expressed during the various stages of prostate cancer (PC). Its chemical inhibition by anti-androgens still represents the first line in PC therapy. Many PCs, however, progress towards a metastatic phenotype. Tumor microenvironment plays a role in PC progression and cancer associated fibroblasts (CAFs) actively interact with epithelial cells, thus fostering tumor progression. The role of AR in stromal cells is unclear.

We have shown that mouse and human fibroblasts express a classical AR that is permanently localized in cytoplasm and does not activate gene transcription upon androgen stimulation of cells. In this location, AR interacts with filamin A (FlnA). Androgens increase AR complexation with FlnA, thus inducing cell motility in the absence of a significant proliferative effect.

Almost all primary CAFs from PC patients with different Gleason's score express AR that is localized in the extra-nuclear compartment. Stimulation of CAFs with androgens does not influence the AR sub-cellular distribution and enhances co-localization of AR with FlnA. Androgens rapidly induce cytoscheleton changes, with the appearance of protrusion and membrane ruffles, typical hallmarks of small GTP-binding protein activation and cell motility. Androgens increase the motility of primary CAFs in both scratch- and transmigration assays. Lastly, a peptide that mimic the region of AR responsible for the receptor interaction with FlnA, prevents cell motility triggered by androgens in primary CAFs. This study suggests that AR expressed in CAFs mediates the recruitment of these cells towards PC epithelial cells upon local androgen concentration increase. New molecules interfering in AR/FlnA complex assembly in CAFs might block this process, thus representing a promising therapeutic approach in PCs.

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Leptin as a mediator of tumor-stromal interactions promotes breast cancer stem cell activity

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Breast cancer is characterized by the presence of a population of cells with stem-cell-like properties (breast cancer stem cells-BCSCs), responsible for tumor initiation, metastasis and resistance to therapies. BCSCs are regulated by complex interactions with stromal cells within the tumor microenvironment. Thus, new therapeutic strategies aimed to target the crosstalk between microenvironment and BCSCs have the potential to improve clinical outcome. We investigated how leptin, as a mediator of tumor-stromal interactions, may affect BCSC activity using patient-derived samples (n=16) and breast cancer cell lines, determining the potential benefit of targeting leptin signaling. Conditioned media (CM) from cancer associated fibroblasts and breast adipocytes significantly increased mammosphere formation in breast cancer cells. Depletion of leptin from CM completely abrogated this effect. Mammosphere cultures exhibited increased leptin receptor (*OBR*) expression and leptin exposure enhanced mammosphere formation. Microarray data revealed a similar expression profile of stem cell-related genes in mammospheres treated with CM compared to leptin. Interestingly, leptin treatment increased BCSC activity in metastatic breast cancer cells isolated from pleural effusions or ascites. The expression of *OBR* and *HSP90*, a target of leptin signaling, directly correlated with mammosphere formation (r=0.68/p=0.05; r=0.71/p=0.036) in metastatic samples. Kaplan–Meier survival curves indicated that *OBR* and *HSP90* expression correlated with reduced overall survival in breast cancer patients (HR=1.9/p=0.022; HR=2.2/p=0.00017). Finally, blocking leptin signaling by using the peptide-LDFI, a full leptin receptor antagonist, significantly reduced mammosphere formation in breast cancer cells and patient-derived samples. Our results suggest that leptin/OBR signaling may represent a potential therapeutic target that can block the stromal-tumor interactions driving BCSC-mediated disease progression.

Role of Hydroxy acid oxidase 2 (Hao2) in hepatocellular carcinoma

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Hydroxy acid oxidases are flavin mononucleotide (FMN)-dependent peroxisomal enzymes capable of oxidizing a broad range of 2-hydroxy acids to 2-keto acids, resulting in hydrogen peroxide formation at the expense of molecular oxygen. Since no data concerning Hao2, a member of this family, and cancer are available in the literature, we analyzed the expression of this enzyme in mouse, rat and human hepatocellular carcinoma (HCC).
Our microarray analysis, performed in the liver of rats subjected to the Resistant Hepatocyte (R-H) model, revealed that Hao2 was among the most down-regulated genes in HCCs. Next, we investigated whether Hao2 down-regulation is an early event during liver carcinogenesis; to this aim, we analyzed the expression of Hao2 by qRT-PCR in preneoplastic lesions and HCCs generated 10 weeks, and 14 months after initiation, respectively. Interestingly, qRT-PCR showed down-regulation of Hao2 already in rat early preneoplastic lesions. To determine whether this down-regulation is a general phenomenon in liver tumorigenesis or is specific only for rat liver, we analyze the expression of Hao2 in a chemically-induced mouse model of hepatocarcinogenesis, consisting of a single injection of DENA followed by treatment with TCPOBOP, that causes massive hepatomegaly and then HCC. Notably, similar to what found in rat HCC, Hao2 was strongly down-regulated also in TCPBOP-induced mouse HCC. Finally, we investigated the expression levels of Hao2 in two distinct series of human HCCs. Interestingly, we found a strong down-regulation of Hao2 gene in human HCCs when compared to both normal and cirrhotic peri-tumoral liver. These results describe that Hao2 is deregulated in HCCs generated in three different species and by different etiological agents and that its down-regulation is a very early event in the development of HCC, and may represent a useful diagnostic tool and a marker of poor prognosis.

Selective and active targeting of neuropeptide Y receptors in experimental oncology: use of gold nanocages conjugated to short peptides

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Background. Neuroendocrine molecules, such as neuropeptide Y (NPY), play a significant role in the development of human cancers, such as breast (BrCa) and prostate (PCa) carcinomas. Interaction between NPY and its Y receptors (Y-Rs) influences cancer progression leading to tumour growth modulation. Y1-R controls cell proliferation, whereas Y2-R promotes angiogenesis. Moreover, a switch from Y2-R expression in normal breast to a Y1-expression in BrCa has been reported. Thus, the development of molecular tools to specifically target Y-Rs are promising for novel diagnostic and therapeutic approaches. Among them, gold nanoparticles are very useful for selective cancer cell targeting due to their low toxicity and simple functionalization with peptides and antibodies leading to high binding affinity and multivalent avidity. Gold nanocages (AuNCs) characterized by hollow interiors, ultrathin and porous walls are of interest due to their strong and highly wavelength-tunable optical absorption in the near-infrared optical window.
Aim. We used AuNCs as a carrier for selective targeting of Y1- and Y2-R in a well-characterized cancer model expressing Y-Rs, the PC-3 human androgen-independent PCa cells. To this purpose, AuNCs have been functionalized with short peptide sequences recognising Y1- and Y2-R.
Results. Au-peptide NCs ability to target Y1- and Y2-R in PC-3 cells was evaluated by confocal microscopy. After 15 min of incubation, only Au-peptide NCs were detected at the cellular membrane. One to 3-h incubation resulted in Au-peptide NCs translocation into the cells. Annexin V and MTT assays did not show any cytotoxic effect driven by Au-peptide NCs.
PC-3 cells treatment with Au-peptide NCs, AuNCs or peptides resulted in a differential pattern of activation of ERK1/2 phosphorylation (Western Blot analysis).
Conclusions. These information will help us to establish whether the presence of AuNCs can positively or negatively affect peptide actions on cancer cell response.

Session IV

Translational Medicine

Epicardial adipocyte hypertrophy: association with M1-polarization and toll-like receptor pathways in coronary artery disease patients

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Background and Aims. In coronary artery disease (CAD) patients epicardial adipose tissue (EAT) shows an elevated inflammatory infiltrate. Toll-like receptors (TLRs) are important mediators of adipose tissue inflammation and they are able to recognize endogenous products released by damaged cells. Because adipocyte death may be driven by hypertrophy, our aim was to investigate in CAD and non-CAD patients the association between adipocytes size of EAT, macrophage infiltration/polarization and TLR-2 and TLR-4 expression.
Methods and Results. EAT biopsies were collected from CAD and non-CAD patients. The adipocytes size was determined by morphometric analysis. Microarray technology was used for gene expression analysis; macrophages phenotype and TLRs expression were analyzed by immunofluorescence and immunohistochemical techniques. Inflammatory mediator levels were determined by immunoassays.
EAT adipocytes were larger in CAD than non-CAD patients and do not express perilipin A, a marker of lipid droplet integrity. In CAD, EAT is more infiltrated by CD68-positive cells which are polarized toward a M1 state (CD11c positive) and presents an increased pro-inflammatory profile. Both TLR-2 and TLR-4 expression is higher in EAT from CAD and mainly observed on CD68-positive cells.
Conclusions Our findings suggested that EAT hypertrophy in CAD patients is associated to M1 infiltrated macrophages which represent the main promoters of local tissue inflammation through TLR-2 and TLR-4 pathways.

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SPECT Molecular Imaging of Melanoma using newly synthetized radiolabeled-RGD antagonists

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Therapies targeting angiogenesis are an integral modality of anti-tumor treatment in many malignancies as colorectal cancer, breast cancer, non small cell lung cancer, glioblastoma and renal cell carcinoma. Among the pro-angiogenic molecules vascular endothelial growth factor (VEGF) has been extensively investigated as a promising target for anti-angiogenic therapy. However, anti-angiogenic treatment with VEGF targeted monotherapy has shown a suboptimal outcome and modest prolongation of progression free survival or overall survival. The development of drug resistance to therapy is the major reason of this failure. Thus, is urgently needed a strategy to address the therapeutic treatment toward an "individualized" therapy based on patient profile characterized by specific biomarkers that can predict outcomes and that can help the clinician to individualize the correct antiangiogenic therapy. Our contribution in this field consisted in developing of new biomarkers of angiogenesis that might be used to stratify patients and predict response to antiangiogenic treatment. We developed Arginine-Glycine-Aspartic acid (RGD) triazole based peptidomimetics antagonist of the αVβ3 integrins, recognized as key factors in tumor angiogenesis. RGD peptidomimetics were characterized for their *in vitro* activity toward integrin αVβ3 expressing melanoma cells, and their structures were designed in view of addressing a longer *in vivo* permanence and optimal pharmacokinetic and pharmacodynamic profiles. Three 125I-radiolabeled compounds have been used as biomarkers for non invasive SPECT/CT preclinical imaging studies in human melanoma xenografts and two of those demonstrated a site specific receptor interaction. Nevertheless further investigation and structure refinement are needed in order to achieve new promising molecular imaging markers of antiangiogenetic therapeutic response.

Space and osteoporosis: how microgravity affects bone microenviroment

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Long-term exposure to microgravity produces side effects among which cardiovascular deconditioning, immune deficiency, muscle atrophy and bone demineralization, all age-related disorders that develop more rapidly in space than on Earth. Bone loss is the most critical disease suffered by astronauts. It begins immediately after arrival in space and, interestingly, it is reversible after return to earth while age-associated osteoporosis is mostly irreversible. Osteoporosis is the result of an imbalance between bone deposition and resorption with consequent decline of bone mass and increased risk of fractures. Bone is a complex highly vascularized tissue, where endothelial cells, human mesenchymal cells (hMSC), hematopoietic progenitors, osteoclasts and osteoblasts all contribute to maintain its integrity.
We focused on studying the cross-talk between human microvascular endothelial cells (HMEC) and primary osteoblasts. We found that simulated microgravity inhibits HMEC proliferation. A protein array performed on the conditioned media from HMEC in simulated microgravity demonstrated a significant increase of TIMP-2 and IL6, both involved in regulating the function of endothelial cells and osteoblasts. We also show that the conditioned media from HMEC cultured in simulated microgravity impact on the proliferation and activity of osteoblasts.
Our future goal is to study the cross-talk between HMEC and hMSC, progenitors of osteoblasts, both in simulated and real microgravity. To this purpose, we already studied the behaviour of hMSC in simulated microgravity. We found an inhibition of hMSC differentiation into osteoblasts upon exposure to simulated microgravity.

Metabolomics: a new tool in the discovery of multiple sclerosis biomarkers and pathogenesis?

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Introduction. Multiple sclerosis (MS) is a chronic disease characterized by a high level of heterogeneity. Metabolomics is an "-omics" approach with the potential to discover new biomarkers. Thus, we investigated the metabolic profiles of MS patients to define the pathways potentially related to its pathogenesis.
Methods. Plasma samples from 73 MS patients and 88 controls (C) were analyzed by ^{1}H-NMR spectroscopy, and followed by multivariate statistical analysis. Discriminant metabolites were identified, quantified and ROC curves calculated. It was possible to correlate these metabolites inside different networks by IPA.
Results. The statistical model obtained identified metabolic differences between the MS and C ($R2X = 0.615$, $R2Y = 0.619$, $Q2 = 0.476$; $p < 0.001$). The differential metabolites included glucose, 5-OH-tryptophan, and tryptophan (lower in MS), and 3-OH-butyrate, acetoacetate, acetone, alanine, and choline (higher in MS). Tryptophan degradation and the energy production were the most relevant canonical pathways and cellular functions obtained. The pathways analysis identifies correlation between tryptophan, glucose and 3-OH-butyrate with immunological/inflammatory disease and neurological disease.
Conclusion. With a metabolomic approach we are able to perform two types of analysis: individualize new potential biomarkers and describe the pathophysiology of multiple sclerosis. The main metabolic changes could be connected to two different metabolic pathways: tryptophan and energy metabolism.

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Serum levels of gelatinases, their inhibitors and neutrophil gelatinase-associated lipocalin: worthwhile non-invasive biomarkers for bladder cancer patients' management

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Recent evidence suggests that neutrophil gelatinase-associated lipocalin (NGAL) is required for the development and/or progression of benign and malignant disease, and is overexpressed in several types of tumor. Gelatinase A and B (MMP-2 and -9) by degrading components of the extracellular matrix and thus promoting the release of growth factors, are important in tumor growth and tumorigenicity. Their activity is strictly controlled by the specific tissue inhibitors: TIMP-1 and -2. Disruption of the balance between MMPs and TIMPs is thought to facilitate tumor progression and recurrence. Moreover, MMP-9 enhances its enzymatic activities by binding NGAL and forming the MMP-9/NGAL complex. Therefore, these six molecules have been proposed as soluble biomarkers for numerous malignancy. We measured the concentration of these molecules in the sera of 41 patients with bladder carcinoma using enzyme-linked immunoassay. Sera samples of 53 healthy volunteers were used as controls. In the sera of the control group, MMP-2, TIMP-2, MMP-9/NGAL complex and NGAL were detected in all specimens, whereas MMP-9 and TIMP-1 were undetectable, being at or low the sensitivity of the assay. Vice versa, all the six molecules were detected in the sera from the patients studied and the mean values were higher than that detected in the control group. In particular tumor staged as non muscle invasive (Ta and T1) showed significantly higher NGAL values compared with muscle invasive (>T1) bladder cancer (32.8 ng/ml *vs* 16.2 ng/ml; p=0.029). The discriminatory ability was confirmed by ROC curve analysis that revealed an AUC of 0.75 with a sensitivity of 0.88 and a specificity of 0.67. These preliminary data suggest that NGAL measurement in sera might provide clinicians additional quantitative and objective information on bladder tumor differentiation.

Session V

Molecular Medicine I
(Infection and Metabolism)

The KIR-ligand HLA-A Bw4 predicts the outcome of hepatitis B infection

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Killer Immunoglobulin like Receptors (KIR) are membrane proteins expressed on Natural Killer cells and on a small subset of CD8 lymphocytes. They influence the activation or inhibition of both cell types through interaction with their ligands, represented by Human Leukocyte Antigen (HLA) class I molecules.
Several studies have shown that KIR/HLA interactions are involved in the pathogenesis and progression of different diseases as viral infections, autoimmune-disorders, or cancer, conditioning susceptibility to or protection against the outcome of the disease.
The aim of this study is to assess whether KIR/HLA interaction is involved in immune response against hepatitis B infection, since HBV infection represents a major health problem with 2 billion people infected and 3 hundred and fifty million people with chronic diseases worldwide.
We are conducting a case-control study in Sicilian population, comparing subjects with chronic hepatitis B with subjects with previous HBV infection (HBc-Ab with or without HBeAb or HBsAb) as controls. A second control population was represented by HBcAb negative subjects.
Peripheral blood samples were collected and genomic DNA was extracted from leukocytes and typed, using PCR-SSP, for KIR and HLA.
Preliminary data showed that HLA-A Bw4 was more frequent in cases than controls (1. cases, 23/35, 66%, vs. HBcAb positive controls, 8/35, 23%, OR 6.47, p=0.0003, and 2. cases, 23/35, 66%, vs. HBcAb negative controls, 6/60, 10%, OR 17.25, p=0.00001). No difference has been found in the frequencies HLA-C1, HLA-C2, HLA-$Bw4^{I}$, and HLA-$Bw4^{T}$; no difference has also been reported in the number of KIRs, both activating and inhibitory, and haplotype A and B between groups.
Our data suggest that the HLA-A Bw4, likely through its interaction with the inhibitory KIR3DL1, is associated to the risk of developing chronic hepatitis B. Data on HBcAb negative subjects suggest also the possibility of selective genetic pressure.

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Botulinum Neurotoxins: from mechanism of action to the development of pan-inhibitors

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Botulinum neurotoxins (BoNTs) are Janus toxins, as they are at the same time the most deadly substances known and one of the safest drugs used by humans. They specifically blocks neurotransmission at peripheral nerve endings through the proteolysis of SNARE proteins, i.e. the essential proteins which constitute the core of the neuroexocytosis machinery. Even if BoNTs are most notably known as seven main serotypes, their actual number is well beyond because each serotype can exist as many different isoforms, with unique immunological properties. This is relevant, as BoNTs are considered potential bio-weapons, and the only one currently available therapy is based on the use of antisera against the seven main serotypes. Nevertheless, all BoNTs isoforms are structurally equivalent and intoxicate peripheral nerve endings with a conserved mechanism, strongly related to their molecular architecture. It consists of two main chains linked together by a unique disulphide bond: the heavy chain (H;100 kDa) which mediates the neurospecific binding, the internalization and the translocation of the catalytic light chain (L;50 kDa). The mechanism consists of five steps: a) binding to nerve terminals, b) internalization via synaptic vesicles, c) pH-driven membrane translocation of L d) interchain disulphide reduction and e) L mediated cleavage of SNAREs. We found that molecules capable of interfere with one of these steps are effective inhibitors of BoNTs activity, *in vitro* as well as *in vivo*. Such chemicals can be considered lead molecules for the development of pan-inhibitors of botulinum neurotoxins, regardless their antigenic variability.

Variable clinical phenotype in homozygous familial hypobetalipoproteinemia due to apoB truncations

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Introduction. Familial Hypobetalipoproteinemia (FHBL) is a co-dominant disorder characterized by plasma levels of LDL-cholesterol and apolipoprotein B (apoB) below the fifth percentile of the general population. FHBL is genetically heterogeneous; in ~ 50% of the cases is due to mutations in *APOB* gene and less frequently, to loss of function mutations in *PCSK9* gene. In most cases, *APOB* gene mutations lead to the formation of C-terminally truncated forms of apoB of various sizes which have a reduced capacity to bind lipids and form lipoprotein particles. Here we report two children with severe hypobetalipoproteinemia found to be homzoygous for novel *APOB* gene mutations.

Material and methods. The first case (HBL-201) was an asymptomatic 13 years-old child incidentally found to have mildly elevated serum transaminases associated with mild hepatic steatosis. The second patient (HBL-96) was a 6 months-old child suspected to have Abetalipoproteinemia, for the presence of chronic diarrhea, failure to thrive, extremely severe hypobetalipoproteinemia and low plasma levels of vitamin E and vitamin A.

Results. The patients were found to be homozygous for two novel *APOB* gene mutations. The HBL-201 child was homozygous for a truncated apoB (2211 amino acids, apoB-48.74) whose size is similar to that of wild type apoB-48 (2152 amino acids) produced by the intestine. ApoB-48.74 is expected to be incorporated into chylomicrons in the intestine but might have a reduced capacity to bind lipids and form secretion competent VLDL in the liver. The HBL-96 child was homozygous for a nonsense mutation (Gln513*) resulting in a truncated apoB (apoB-11.30) which is probably degraded intracellularly. In this child the impaired chylomicron formation is responsible for the early clinical manifestations and the growth retardation.

Conclusions. In homozygous FHBL the capacity of truncated apoBs to form chylomicrons is the major factor which affects the severity of the clinical manifestations.

Spectrum of mutations of the Lipoprotein Lipase (LPL) gene and other candidate genes in primary hypertriglyceridemias

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Background. Monogenic hypertriglyceridemia (HTG) may result from mutations in a set of five major genes which impair the intravascular lipolysis of triglyceride (TG)-rich lipoproteins mediated by the enzyme Lipoprotein Lipase (LPL). Mutations in the *LPL* gene are the most frequent cause of monogenic HTG (familial chylomicronemia) with recessive transmission and an estimated prevalence of $1:10^6$. Severe HTG is associated with the risk of recurrent severe pancreatitis.
Methods. The *LPL* gene and other candidate genes were resequenced in 149 patients with severe HTG (TG >10 mmol/L) and 106 patients with moderate HTG (TG >4.5 and <10 mmol/L) referred to tertiary Lipid Clinics in Italy.
Results. In the group of severe HTG, 26 patients (17.4%) were homozygotes, 9 patients (6%) were compound heterozygotes and 15 patients (10%) were simple heterozygotes for rare *LPL* gene variants. Single or multiple episodes of pancreatitis were recorded in 24 (48%) of these patients. There was no difference in plasma TG concentration between patients with or without a positive history of pancreatitis. Among moderate HTG patients, six patients (5.6%) were simple heterozygotes for rare *LPL* variants; two of them had suffered from pancreatitis. Overall 36 rare *LPL* variants were found, 15 of which not reported previously. Systematic analysis of close relatives of mutation carriers led to the identification of 44 simple heterozygotes (plasma TG 3.2 ± 4.1 mmol/L), none of whom had a positive history of pancreatitis. Among severe HTG patients, five subjects were found to be homozygous or compound heterozygotes for mutations in other lipolysis related genes (*APOA5* and *GPIHBP1*).
Conclusions. The prevalence of rare *LPL* variants in patients with severe or moderate HTG, referred to tertiary lipid clinics, was 50/149 (33.5%) and 6/106 (5.6%), respectively. Systematic analysis of relatives of mutation carriers is an efficient way to identify heterozygotes who may develop severe HTG.

Notch3-induced deregulated CXCR4 expression in acute T cell lymphoblastic leukemia model

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Notch deregulated signaling is one of the major cause of acute T cell lymphoblastic-leukemia (T-ALL) in humans and mice. The oncogenic function of Notch3 in T-ALL was demonstrated by a murine model of our laboratory, characterized by enforced expression of the Notch3 active form (N3-IC) in immature thymocytes (N3-ICtg). Deregulated proliferation and maturation at the preT/T transition phase and constitutive activation of preTCR were observed in N3-ICtg mice.
Multiple signals from stroma sustain T cell differentiation programs in the thymic niche. Cooperative signaling among the preTCR, CXCR4 and Notch are required at β selection for the continued differentiation from Double Negative (DN) to Double Positive (DP) T cells. The stromal cell derived factor SDF-1(CXCL12) and its receptor CXCR4 promote survival of DN thymocytes and regulate the migration during the DN/DP transition. The CXCR4/SDF-1 axis has been suggested to play a role in the pathogenesis of T-ALL.
In Notch3-ICtg mice, DP-gated thymocytes display an increased level of CXCR4 surface expression per cell with respect to wt. Furthermore, most of the DP thymocytes highly co-express Notch3 and CXCR4. Abnormally represented DP T cells found in blood, spleen and lymph nodes show a combined expression suggesting a crosstalk of the two receptors. Moreover, T/DP cells were over-represented in bone marrow of N3-ICtg mice, all characterized by an increased and combined Notch3/CXCR4 expression. Inhibiting activation (GSI treatment) or interfering on the expression of Notch3 (shRNA) we could modulate CXCR4 surface expression in a CD4+/CD8+/CD3+ human leukemic cell line, TALL1. Our results are suggestive of Notch3 deregulated pathway that may modulate DP cells egress from thymus, in early steps of T-ALL development, by forcing CXCR4 surface expression through an *erk*-mediated mechanism.

Session VI

Molecular Medicine II

(Pathophysiology)

Bone marrow B cell clones expanded in cryoglobulinemic patients with and without nephritis expressed distinctive CDR3 sequences with rheumatoid factor activity

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In a recent study investigating the relationship between the pattern of B-cell expansion in the bone marrow (BM) and cryoglobulinemic vasculitis (CV) clinical manifestations, a significant association with glomerulonephritis was observed, suggesting that preferential expansion of particular clones may be implicated in the development of renal disease.
The present study was aimed to investigate B-cell clonal expansion specifically related to the renal involvement in CV.
BM B cell clonality was investigated by amplification of the hypervariable CDR3 fragment of the VH chain, identifying clonal B-cell expansion by the presence of one (monoclonal) or few (oligoclonal) narrow band(s) within the predicted size range. In 19 B-cell clonal patients (9 with and 10 without nephritis) further specific sequence analysis were made by bacterial vector subcloning. At least ten colonies were sequenced for each patient, identifying the dominant clone as the most represented one and the other less represented as the minority clones. All the in frame sequences recognized by IMGT as IgG productive clones were aligned by multiple sequence alignment with hierarchical clustering (Multalin).
Six out of nine (66.7%) patients with nephritis, disclosed a B-cell clonal expansion, dominant in two cases, characterized by a significant sequence homology of the D fragment of the hypervariable IgH region, resembling rheumatoid factors (RF), such as MR20 and C93. In contrast, in patients without nephritis, five out of ten (50%) express a B-cell clone characterized by a sequence homology of the CDR3 fragment with RF WOL. Both MR20 and WOL were previously associated to overt B-cell lymphoma clones described in patients affected by CV.
In conclusion, a specific antigen-binding region in B-cell clones with RF activity may be associated with the development of different clinical manifestations in CV.

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Activated monocytes and M1-like macrophages release the b-GGT fraction of gamma-glutamyltransferase that accumulates in atherosclerotic plaques

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Gamma-glutamyltransferase (GGT) is a well-established independent risk factor for cardiovascular mortality related to atherosclerotic disease. Four GGT fractions have been identified in plasma, but only b-GGT fraction was detected in atherosclerotic plaques, correlating with the histological markers of vulnerable plaque.
The present study was aimed to evaluate whether macrophage lineage cells may constitute a source of b-GGT within the atherosclerotic plaque. Human monocytes were isolated from peripheral blood of healthy donors. GM-CSF and M-CSF were used to induce differentiation into M1-like and M2-like macrophages, respectively. We found that M1-like macrophages express higher levels of GGT as compared to M2-like, and that both monocytes and M1-like macrophages – but not M2-like – are able to release b-GGT fraction upon activation with pro-inflammatory cytokines. Western blot analysis of b-GGT extracted from plaques confirmed the presence of a GGT immunoreactive peptide compatible with that of macrophages.
Our data thus indicate that macrophages characterized by a pro-inflammatory phenotype may contribute to intra-plaque b-GGT accumulation, which in turn may play a role in atherosclerosis progression by modulating inflammatory processes and favouring plaque instability.

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The key roles of ERBB2 in cardiomyocytes proliferation and heart regeneration

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Heart injuries such as those induced by acute ischemia can lead to heart failure, the most common cardiac ailment and a serious health problem worldwide. This occurs mainly due to the inability of the mammalian heart to regenerate after injury.
The growth factor Neuregulin (NRG1) and its tyrosine kinase receptors ERBB4 and ERBB2 play essential roles during heart development. NRG1 administration improves cardiac function in injured mice and in heart failure patients. Developing strategies to boost NRG1-induced cardiac regeneration processes in humans is therefore clinically imperative.
We recently unveiled that NRG1-induced cardiomyocytes proliferation diminished 1 week after birth due to a sharp reduction in ERBB2 expression. Using loss- and gain-of-function genetic tools, we explored the role of ERBB2 in cardiac growth and regeneration.
Cardiomyocytes-specific Erbb2 knockout revealed that ERBB2 is required for cardiomyocytes proliferation at embryonic/neonatal stages.
On the other hand, cardiomyocyte-specific induction of a constitutively active ERBB2 (caERBB2) in neonatal, juvenile and adult mice generated pronounced cardiomegaly characterized by extensive cardiomyocytes dedifferentiation, proliferation and hypertrophy.
Transient induction of ERBB2 signalling in juvenile or adult mice stimulated cardiomyocyte dedifferentiation and proliferation and allowed anatomical and functional cardiac regeneration following ischemic injury. Thus, augmentation of ERBB2 signalling awakes cardiac regenerative ability in mice.
We are currently working on the optimization of ERBB2 signalling as a promising therapeutic approach to cardiomyocytes replacement in heart failure.

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B7h triggering inhibits osteoclast function in vitro and in vivo

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Hyperactivation of osteoclasts (OCs) can be detected in conditions such as osteoporosis, rheumatoid arthritis and other autoimmune diseases, and osteolytic tumor metastases. B7h is the ligand of the ICOS T cell costimulatory molecule, and it is expressed in haematopoietic and non-haematopoietic cells. Recent reports have shown that the B7h:ICOS interaction may trigger bidirectional signals which are able to modulate the response of both the cells expressing ICOS and those expressing B7h. This work gems from our finding that OCs (differentiated *in vitro* from monocytes by culture in the presence of RANKL and M-CSF) can express B7h, and it was aimed to investigate the effect of B7h triggering on differentiation and function of OCs *in vitro* and *in vivo*. The *in vitro* results showed that B7h triggering using ICOS-Fc (a recombinant soluble form of ICOS) reversibly inhibited OCs differentiation from monocytes in terms of acquirement of the OCs morphology (giant multinuclear cells) and the $CD14^{-}$Cathepsin $K^{+}TRAP^{+}$ phenotype. Moreover, it induced reduction of the size of cells and nuclei, decreased ability to adhere to the substrate and to promote calcium release from crystalline calcium phosphate. A similar effect was detected on already differentiated OCs. The *in vivo* results showed that the treatment with ICOS-Fc strikingly inhibited the systemic bone resorption induced in mice by treatment with high doses of soluble RANKL, which is a mouse model of osteoporosis. Therefore, these data detected a novel molecular system involved in osteoimmunology. Moreover, they open a novel field in the pharmacological use of agonists and antagonists of the ICOS/B7h system which to date have been envisaged as immune modulators mainly in the fields of autoimmune diseases and anti-tumor immune response.

POSTERS

Are table green olives nutraceuticals foods?

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Mediterranean Diet is the typical diet of the countries of the Mediterranean basin that have in common, as key elements, olives and extra virgin olive oil. It is now clear that olive oil, as a main source of fat, plays a key role on the control of oxidative stress and inflammation, whereas little is known about the role of table green olives.
We have performed a nutritional intervention, administering to 25 healthy subjects 12 table green olives/day, containing $1{,}5x10^6$ CFU/g *Lactobacilli* spp. for 30 days. The olives, in salt solution, belong to the variety Nocellara del Belice of Campobello di Mazzara (TP). A food questionnaire (EPIC Questionnaire) was given to each of them, in order to evaluate their eating habits.
The aim of this study was to analyze the nutraceutical properties of this traditional food. In particular, we have analyzed the quantitative changes of the gut microbiota in healthy voluntary subjects. Emerging evidences, in fact, underline the association between gut microbiota and food as the basis of many phenomena that affect health and delay or avoid the onset of some diseases. We also carried out biochemical, oxidative stress and cytokines analyses.
The preliminary results show a quantitative variation of some molecules as Malondialdehyde and Paraoxonase, linked to oxidative stress, confirming that these olives could have an anti-oxidant effect. In some subjects the amount of *Lactobacilli* spp in feces increases, demonstrating that the olives have a possible probiotic-like effect and stimulate the growth of bacteria that contribute to eubiosis of the gut microbiota. In addition, the level of interleukins seems to vary, demonstrating how this food is also able to modulate the inflammatory response (as previously shown by studies conducted on extra virgin olive oil).
In conclusion, these preliminary results suggest a nutraceutical effect of daily olive consumption.

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Gliadin modulates epithelial permeability by inducing M2 activation in macrophages

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Celiac disease (CD) is a chronic small intestinal enteropathy due to ingested gluten in genetically predisposed individuals. A complex interplay between adaptive and innate responses has been described in CD, although the link between the activation of immune cells and the epithelial defects in affected intestine is thus far not clarified. Recently, a role for arginine and its metabolites in gluten-triggered immune responses has been described; here we address the role of macrophage derived arginine metabolites in the affection of epithelial permeability.
RAW264.7 macrophages were incubated for 24h with 1mg/ml PTG, obtained through enzymatic digestion of gluten with pepsin and trypsin. The induction of arginine metabolism by iNOS was monitored by measuring nitrite (stable derivatives of NO) in the extracellular medium, while arginase activation was evaluated by means of UHPLC-MS/MS in terms of polyamines release. After the treatment, aliquots of macrophages incubation medium were added to the basolateral side of polarized Caco-2 intestinal epithelial monolayers; their integrity was monitored by measuring transepithelial electrical resistance (TEER).
The treatment of macrophages with PTG caused a significant induction of both iNOS and arginase expression, paralleled by a consistent accumulation of nitrite and polyamines in the extracellular medium. In turn, the addition of incubation medium from treated macrophages to the epithelial monolayers caused a significant decrease of TEER, index of increased permeability. This effect was efficiently reduced when conditioned medium added to epithelial cells was obtained by treating macrophages with PTG in the presence of the arginase inhibitor DFMO or in arginine-free medium, hence indicating that PTG-dependent secretion of polyamines by macrophages may modulate intestinal permeability *in vitro*. Whether this also occurs in vivo deserves to be further investigated, as well as the specific role of macrophages in the intestinal alterations typical of CD patients.

^{111}InDOTA-aminoproline-RGD based cyclic semipeptides (cAmpRGD) for SPECT Molecular Imaging of Melanoma

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The role of integrins in the regulation of angiogenesis is an essential feature in tumor progression and metastatic dissemination. Among the many different classes of integrins, defined by their subunit composition and binding specificity, $\alpha V\beta 3$ are recognized as key factors in tumor angiogenesis and many efforts have been dedicated to integrins as selective targets in molecular imaging of cancer. Integrin-targeted radiotracers are putatively able to reveal the presence of a tumor mass and to monitor the growth and diffusion of tumor cells. In this view, we evaluated the chemical and biologic properties of novel aminoproline-RGD based cyclic semipeptides (cAmpRGD). We developed a suitable labeling protocol to covalently conjugate these cyclic AmpRGD semipeptides with DOTA chelating unit. We used pre-clinical Single Photon Emission Computed Tomography (SPECT) for *in vivo* imaging of ^{111}In-labelled DOTA-cAmpRGD compounds.

We found that AmpRGD semipeptides inhibited human melanoma cells and human bone marrow-derived endothelial precursor cells (EPCs) adhesion to Arg-Gly-Asp-(RGD) containing substrata. Moreover AmpRGD semipeptides strongly inhibited in vitro capillary morphogenesis of EPCs. The biodistribution studies, performed in healthy CD1 nu/nu mice, revealed a rapid blood clearance of the labelled compound and the involvement of renal excretion pathway with only a marginal involvement of liver excretion pathway. Displacement experiments performed in CD1 nu/nu mice bearing human melanoma xenografts confirmed intratumoral uptake and specific binding activity of the ^{111}In-labelled DOTA-cAmpRGD compound.

These preliminary results suggest that cAmpRGD bioconjugates are promising candidates for non-invasive SPECT imaging of highly angiogenic cancer lesions.

ERAP1 is a novel drug target in the oncogenic Hedgehog signaling pathway

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Endoplasmic reticulum aminopeptidase ERAP1 is essential for the maturation of a wide spectrum of peptides and is involved in several biological functions such as antigen processing, cytokine receptor shedding and neo-angiogenesis. ERAP1 enzymatic activity contributes to the pathogenesis of several major human diseases ranging from viral and parasitic infections to autoimmunity and cancer. Recently, inhibition of ERAP1 peptide trimming has been shown to play a key role in stimulating innate and adaptive anti-tumor immune responses. Here, we show a new biological role for ERAP1 in a not-immune mediated control of cancer. We demonstrate that inhibition of ERAP1 by RNA interfering or pharmacological treatment, increases the repressor form of the transcription factor Gli3, thus impairing the activation of Hedgehog signaling, an essential pathway in both development and tumorigenesis. Notably, both pharmacological and genetic inhibition of ERAP1 reduce Hedgehog-dependent tumor cells growth *in vitro* and in allograft Medulloblastoma model *in vivo*. These data identify a novel molecular mechanism in the regulation of Hedgehog signaling and strongly support ERAP1 as a novel drug target in this oncogenic pathway.

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CD133 affects the architecture and dynamics of plasma membrane protrusions, and modify the expression of key players involved in cell polarity and migration in a colon cancer cell line

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CD133 is a transmembrane glycoprotein considered a marker of colon cancer stem cells, confined to lipid microdomains enriched in cholesterol. The cytoplasmic tail could potentially interact with the cytoskeleton, by changing the dynamics of plasma membrane protrusions. The dynamic of actin-membrane protrusions is involved in cell polarization and migration. Aim of this study was to evaluate the molecular and functional effect of the overexpression of CD133 full-length (HCT-CD133) on the architecture and dynamics of plasma membrane protrusions in HCT116 colon cancer cells, compared to the overexpression of a CD133 variant deleted of its C-terminal domain (HCT-CDdel). The cDNA encoding the full length CD133 molecule and its variant deleted of the C-terminal domain were cloned into the expression vector pCDNA3 and transfected into HCT116 cells. The number and length of plasma membrane protrusions were evaluated by Scanning Electron Microscopy. For analysis of actin organization the cells were stained with phalloidin, and the actin fibers Coherency was calculated. Electric Cell-substrate Impedance Sensing was used for analysis of motility. RT-PCR and WB analyses were used to analyze changes in mRNA and protein expression levels occurring in these cells. HCT-CD133 displayed long protrusions on cell surface while cells transfected with the C-terminal deleted variant displayed abnormal membrane structures. The HCT-CD133 cells also displayed an increased cell motility compared with HCT-CDdel and vector control cells. Moreover, actin was more disorganized in HCT-CD133 cells compared to HCT-CDdel and CD133 overexpression was associated with an increased expression of key players of actin cytoskeleton such as CRMP4, Rho GTPases and VEGFA. These results suggest an involvement of the CD133 molecule in regulating the architecture of plasma membrane protrusions which warrants further studies to understand the potential implications in terms of signal transduction and membrane trafficking events.

Modulation of exosomes secretion during NaBu Induced Differentiation of HT29 Colon Cancer Cells, and effects of exosomes administration on normal and cancer cells

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Differentiation of normal and cancer stem cells is one of the most important issue in biology. CD133 is a surface protein marker of colon cancer stem cells shown to be released by exosomes upon differentiation of colon cells. Exosomes are small extracellular vesicles and are a tool for extracellular communication. Our group previously reported that sodium butyrate (NaBu)-induced differentiation of HT29 colon cancer cells is associated with a reduced expression of CD133. Aim of this study was to analyze the effects of NaBu administration on exosomes release and protein cargo in HT29 cells. The exosome fraction was prepared using differential centrifugations by cells undergoing NaBu-induced differentiation. The correct isolation of exosomes was confirmed by Dynamic Light Scattering, Electron microscopy, and Western-Blot analysis. The Bradford assay was used for the quantitative evaluation of isolated exosomes. Protein and mRNA expression levels were evaluated by western blot and RT-PCR analyses, respectively. The uptake of exosomes was analyzed by confocal microscopy. We found that exosomes released during NaBu-induced differentiation are enriched in CD133. This effect was specific since it did not involve other membrane proteins. Interestingly, incubation with exosomes isolated from differentiating HT29 cells increased the proliferation rate of both normal and tumor cells, increased the colony-forming efficiency of cancer cells and reduced the NaBu-induced differentiation of HT29 cells. Such effects were associated with an increased phosphorylation level of both Src and Erk proteins. These findings confirm the role of CD133 in the maintenance of stem cells properties, and that cancer cells-derived exosomes are able to significantly affect the behavior of recipient cells.

Aberrant expression of PTEN in squamous lung cancer cells: molecular analysis and impact on the synergistic combination of the targeted therapy with BKM120 and Defactinib

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Squamous cell carcinoma of the lung (SQCLC) accounts for approximately 30% of non–small cell lung cancer (NSCLC) and it is almost invariably associated with smoking. Recent efforts to identify molecular "oncogenic drivers" of SQCLC demonstrated that key proteins of the PI3K/AKT/mTOR pathway are frequently deregulated in SQCLC: loss/reduction of PTEN level occurs approximately in 70% of SQCLC, pointing its relevant role in cancer progression. The PTEN protein regulates several physiological processes such as cell survival, cell growth, cell migration and focal adhesion by inhibiting the PI3K/AKT pathway and focal adhesion kinase (FAK) signaling. The aim of this study was to evaluate how reduced PTEN levels affect the biological properties of SQCLC cells and to investigate the activity of specific agents targeting the altered pathways in these cell clones. The reduction of PTEN levels promotes cell growth by activating both focal adhesion kinase (FAK) and AKT, with enhanced cell migration and invasiveness, associated to increased levels of metalloproteinase 2 and mir-21. Cells harboring low PTEN expression are more sensitive to pan-PI3K class I inhibitor NVP-BKM120 compared to control cells in term of cell viability and cell migration, as confirmed by reduced AKT activation and MMP2 expression, respectively. Despite FAK activation, we failed to observe any increased response to Defactinib, in term of both cell viability and cell migration. Finally, we tested the effect of the NVP-BKM120/Defactinib combination. In spite of the low selectivity of Defactinib alone, synergistic effects in term of cell viability and migration was observed in cells harboring low PTEN expression while only a weak additive effect was observed in the parental cells. We can conclude that reduced PTEN levels stimulate cell proliferation and migration and that the use of pan-PI3K class I inhibitor in association with a FAK inhibitor could be a promising strategy to target cancer cells with this genetic alteration.

TERT inhibition leads to cell cycle alterations and increases the apoptotic effects of chemotherapeutic agents in EBV-immortalized B lymphocytes

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Background. Besides its canonical role in stabilizing telomeres, Telomerase Reverse Transcriptase (TERT) may promote tumorigenesis through extra-telomeric functions. Lymphoblastoid cell lines (LCLs), generated by EBV infection of B lymphocytes, are a useful *in vitro* model to investigate post-transplant lymphoproliferative disorders and B-cell lymphomas. BIBR1532 (BIBR), a powerful TERT inhibitor, has been evaluated in several cell lines, but no data are available in EBV-driven B-cell malignancies. Our aim was to investigate the effects of BIBR on LCLs alone or in combination with Fludarabine (FLU) or Cyclophosphamide (CY), chemotherapeutic agents employed in the treatment of B-cell malignancies.
Methods. We employed LCLs at early and late passages of culture after EBV infection, with low and high levels of TERT expression respectively and LCL expressing ectopic TERT. TERT levels and activity were assayed by real-time PCR and TRAP assay. Apoptosis and cell cycle profiles were evaluated by flow cytometry. Additional experiments were conducted *in vivo* on zebrafish.
Results. BIBR selectively inhibited telomerase activity in TERT-positive LCLs. TERT inhibition led to a decrease (57%±3%) of cell proliferation and an increase (23%±2%) of apoptosis, compared to untreated cells. In addition, BIBR modified the cell cycle profile with an accumulation of cells in the S phase. Experiments on zebrafish confirmed the results obtained *in vitro*; BIBR led to accumulation of cells in the S phase and higher apoptosis (227%±3%) compared to untreated embryos. TERT-positive LCLs treated with BIBR+FLU or BIBR+CY showed a significant increase of apoptotic cells, compared to cell treated with chemotherapeutic agents alone (34% *vs* 15% p=0.0051 and 68% *vs* 46% p=0.0062, respectively).
Conclusions. TERT inhibition affects the progression of the cell cycle and increases cell susceptibility to FLU and CY. The results suggest new therapeutic applications of TERT inhibitors in EBV-related malignancies.

Phosphatidylserine-dependent antiprothrombin antibobies in clinical practice

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Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by vascular thrombosis and/or pregnancy morbidity and the persistent presence of antiphospholipid antibodies (aPL). Several recent papers underlined the relevant role of the anti-prothrombin/phosphatidylserine antibodies (aPS/PT) in APS diagnosis [Sciascia et al. Thromb Haemost. 2014]. Of note, aPS/PT were recently recommended as a surrogate of LA to overcome the technical limitations of the functional assay [Pregnolato et al. Immunol Res. 2013]. We introduced the immunosorbent aPS/PT antibodies assay (Quanta Lite, Inova Diagnostics, Inc.) as a routine anti-phospholipid (aPL) test in our diagnostic laboratory in march 2013. So far (October 2014) we identified 265 aPS/PT positive patients comprising 143 LA positive, 93 LA negative and 29 LA undetermined patients (among which 11 in oral anticoagulant therapy - OAT). LA positive patients presented IgG±IgM aPS/PT positive antibodies in 47 (32.9%) cases while only IgM in 96 (67.1%). In contrast, LA negative patients presented 57 (61.3%) IgG±IgM aPS/PT positive cases and 36 (38.7%) IgM cases. 37 (25.9%) LA positive patients were also aB2GPI positive, while only 4 (4.3%) LA negative patients were also aB2GPI positive. Among patients in OAT (six lupus patients with APS and five with venous or arterial thrombosis) only one resulted also aB2GPI positive. Sixty-eight patients were confirmed as aPS/PT positive in two or more subsequent determinations. In our hands, testing also for aPS/PT antibodies allowed to finally re-classified a significant percentage of patients, previously negative for the classic aPLs. The introduction of aPS/PT antibodies in the diagnostic process of APS is highly recommended, since they disclosed diagnostic laboratory performances at least equal to the aCL and aβ2GPI antibodies and a high correlation with LA activity, such that they can be a viable alternative.

Analysis of the Notch-signaling Duality

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Epithelial cancer is one of the most important cancer worldwide in terms of lethality and frequency, in particular squamous cell carcinoma (SCC) is one of the predominant type in skin and oral cavity context. Thinking to the genetic basis of epithelial cancer, the major set of genes mutated are TP53, NOTCH1, CDKN2A, EGFR and HRAS. Great interest has come by mutation in Notch1, a master regulator of skin differentiation, because the importance of Notch1 has been improved by the growing body of data from whole genome analysis that puts it as a central driver mutation. The specific role of NOTCH1 seems controversial because has been described as oncogene and tumor suppressor gene. We previously showed that the mechanism for the dichotomous function of Notch signaling in cancers might be substantially influenced by the cellular signaling context under which the receptor is expressed rather than the cellular type context. In our study we have explored the mechanisms underlying the effect of Notch1 through a RNA-Seq analysis of SCC derived cells after GSI inhibition. We found both positive and negative Notch1 SCC-tumors. In Notch1-positive tumors we revealed a subset of Notch1 regulated genes which function is required for SCC tumor phenotype. Interestingly, in these cells Notch/Hes pathway is defective, indicating that molecular mechanism act in silencing specific arm of the Notch response. Our work demonstrates that the molecular duality of the Notch pathway represent an intrinsic characteristic of Notch signaling, and we provide evidence on how cellular molecular mechanisms control Notch response.

Extra virgin olive oil phenolic compounds modulate inflammatory response in human intestinal Caco-2 cells treated with dietary oxysterols

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Experimental studies suggest a dietary intake of compounds with antioxidant action such as phenolics as beneficial for human health. In particular, extra virgin olive oil phenolic compounds reach the highest concentration in intestinal lumen before absorption. Therefore, these molecules can display their strong antioxidant and anti-inflammatory actions in the intestine rather than in other tissues. The aim of this study was to evaluate the ability of olive oil phenolic compounds to counteract the pro-oxidant and pro-inflammatory actions of a mixture of oxysterols, which can be present in a cholesterol-rich diet. Differentiated human intestinal Caco-2 cells were pre-treated with a monovarietal extra virgin olive oil phenolic extract obtained from the Cultivar Bosana, the most common and widespread in Sardinia, and then treated with the oxysterol mixture. Oxysterols treatment, depending on time of exposure and concentrations used, up-regulated two key inflammatory interleukins, IL-6 and IL-8, through the production of reactive oxygen species (ROS) and modulation of MAPKs (p38, JNK and ERK). Bosana phenolic extract, rich in the biologically active hydroxytyrosol, tyrosol and their secoiridoids derivatives, was able to significantly counteract oxysterols intestinal damage. Protective effect of olive oil phenolic compounds is likely due to their both direct antioxidant action as free radical scavengers, and indirect action as modulators of specific intracellular transduction molecules involved in pro-oxidant and pro-inflammatory signals triggered by dietary oxysterols.

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Oncogenic role of the E545K mutant form of the p110 catalytic subunit of phosphoinositide 3-kinase (PIK3CA) in the mouse liver

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Objectives. Phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha isoform (PI3KCA) is frequently mutated or overexpressed in various human cancers, including hepatocellular carcinoma (HCC). Here, we addressed the oncogenic role of a mutant form of PI3KCA (PIK3CA-E545K) and investigated its downstream effectors in the mouse liver.
Methodology. The PIK3CA mutant E545K was overexpressed in the liver of 129/Sv-C57BL/6 mice via hydrodynamic transfection. Histopathological analysis of liver lesions developed in PIK3CA-E545K mice was conducted three and six months after hydrodynamic gene delivery. Microarray analysis of the same lesions was performed to identify putative PIK3CA targets.
Results. Overexpression of PIK3CA-E545K in the mouse liver triggered the development of liver preneoplastic lesions and hepatocellular tumors 3 and 6 months, respectively, after hydrodynamic transfection. Microarray analysis showed the overexpression of genes involved in lipid metabolism, cell proliferation, and survival in preneoplastic and neoplastic lesions of PIK3CA-E545 mice when compared with control mice.
Conclusions. The present study indicates that oncogenic forms of PIK3CA are sufficient to induce malignant transformation of the mouse liver. Also, we identified a number PIK3CA targets in the liver, whose investigation might be helpful both to better understand the molecular pathogenesis of HCC and for the development of new therapeutic strategies against this deadly disease.

Androgens trigger neuritogenesis through a cross talk between androgen receptor and TrkA in PC12 cells

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The nervous system (NS) represents one of the major target of sex steroids. These hormones contribute to sex-dependent regulation of neuron and glial cells and protect neurons against injury. In particular, androgens regulate sexual, reproductive and aggressive behaviors in NS, but also control several cognitive abilities. Androgens also induce the formation of an appropriate neuronal network regulating the dendritic branching and axonal growth during development and repair of NS. The androgen mode of action in nervous system is however still unclear.

We here report that rat adrenal pheochromocytoma PC12 cells, a model widely used to study neuronal differentiation, harbor low levels of classic AR, which is predominantly localized in the extra-nuclear compartment of PC12 cells and does not activate gene transcription, regardless of androgen stimulation. In PC12 cells, AR mediates both androgen and NGF-induced neurite elongation. AR inhibition by bicalutamide or depletion by specific siRNA interferes in neuritogenesis stimulated by androgens or nerve growth factor (NGF), suggesting a role for AR not only in androgen, but also in NGF signaling. In turn, a pharmacological TrkA inhibitor interferes in NGF- or androgen-induced neuritogenesis. The cross talk between AR, filamin A (FlnA), and TrkA regulates this process. Once assembled, the tripartite complex activates the downstream PI3-K δ/Rac pathway, which impinges on neuronal differentiation. In PC12 cells stimulated with androgens or NGF, β1 integrins link TrkA with AR and control the upstream events leading to neurite outgrowth and elongation.

These findings imply a new and unexpected role for AR in neuritogenesis induced by androgens or NGF and suggest that specific targeting of AR non-genomic functions in neurons may be useful in the therapy of neurodegenerative disease.

Extra-virgin olive oil and health: food effects on oxidative stress

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Extra-virgin olive oil (EVOO) is an important food in Mediterranean diet (MeDiet), which is considered one of the healthiest dietary models for its beneficial effects on health and wellness. Nutritional epidemiological studies suggest an inverse relationship between coronary heart diseases and consumption of MeDiet food.
EVOO represents the main source of dietary fat in MeDiet, provides high oleic acid content and polyphenols, which have an anti-oxidant activity, exerting anti-inflammatory and anti-atherogenic effects, reducing the concentration of the oxidized low density lipoprotein (oxLDL).
A number of studies in vitro and in animal models have suggested that olive oil polyphenols are able to bind to LDL and this may increase their resistance to oxidation.
Due to the importance of olive oil in MeDiet and its relevance in the economy of Sicily, our group recruited healthy young and old people to highlights the nutritional properties of EVOO and in particular of its polyphenols on the immune-inflammatory and oxidative stress responses.
We have performed a nutritional intervention, administering to healthy young and old subjects 30g olive oil/day for 30 days. A food questionnaire (EPIC Questionnaire) was given to each of them, in order to evaluate their eating habits.
A sample of peripheral blood was obtained as baseline and after 30 days. Sera were frozen and analyzed all together. We have analyzed the quantitative changes of serum levels of oxidative stress markers and inflammatory cytokines. Variation were observed between baseline values and post-treatment values taking into account age and gender.
The results of the study seem to confirm the nutraceutical properties of EVOO that will be useful in the prevention of inflammatory diseases.

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Mechanism of hormone resistance associated with estrogen receptor mutations in breast cancer

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Background. The idea that somatic estrogen receptor gene (ESR1) mutations could play an important role in the evolution of hormone-responsive breast cancers was proposed by us with our original identification of two ESR1 mutations at residues K303 and Y537. Recently, Next Generation Sequencing of metastatic tumors, revealed the presence of ESR1 mutation in metastatic tumors, confirming our previous hypothesis. ESR1 mutant allele frequencies vary over a wide dynamic range, and are usually a minority population within tumors. Therefore, we address how that minor subclonal tumor population drive resistance in metastatic tumors.
Methods. MCF-7 cells expressing endogenous wild-type ER was transduced with ESR1 mutants, Y537N, Y537S, and D538G lentivirus and stable clones selected. ER transcriptional assays, growth in soft agar, duolink, co-immunoprecipitate and western blot assays were performed.
Results. Mutant ER constitutive transcriptional activity was fully antagonized by the antiestrogens tamoxifen or fulvestrant in MCF-7 stable transfectants. In contrast, soft agar growth of all ESR1 mutant-expressing cells was unexpectedly and completely resistant to the growth inhibitory effects of tamoxifen, although mutant-expressing cells were a minority subpopulation in the stable clones. Therefore, in cells with WT ER co-expression, the mutant resistant phenotype dominates. We found that activation of IGF1Rß signaling was involved in all ESR1-mutant resistant cells. Treatment with a specific IGF1Rβ inhibitor in combination with tamoxifen drastically restored hormone sensitivity in cells expressing the ESR1 mutations.
Conclusions. We hypothesize that the selection of dominant-acting ESR1 mutations in tumors is a key event in breast cancer progression, potentially due to the selective pressure of antiestrogens. The dominant-resistant phenotype of ESR1 mutants in a majority WT background supports the subclonal evolution of ESR1 mutations in breast cancer recurrence. Constitutive IGF1Rβ activation is a common resistance mechanism. Blocking this pathway in ESR1 mutation-positive metastatic patients it could be considered as a feasible clinical goal.

Notch3 deregulation induces proliferative disease with lympho/myeloid features

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Notch receptors play an important role in both T cell differentiation and T cell leukemia development. Notch3 activation inside T cell compartment of transgenic (*N3tg*) mice induces an acute form of T cell leukemia (T-ALL). Recently, a role of Notch signaling deregulation in some subset of myeloid tumors expressing T cell markers was suggested, and conversely, the overexpression of myeloid genes in some T-ALL clusters has been described. To study the effects of a T-cell specific deregulation of Notch on inducing the acquisition of myeloid features inside T tumoral cells, we purified $CD4^+CD8^+$ (DP) immature T cells from spleen, bone-marrow and thymus of *N3tg* mice and from thymus of *wt* controls. Real-time RT-qPCR assays revealed in these cells the overexpression of genes involved in myeloid differentiation (e.g. CEBPalpha, MPO), normally silent in DP T *wt* cells, that was confirmed at protein level, by Western blotting and/or FACS experiments. We also identified a subset of DP *N3tg* T cells showing intracellular co-expression of MPO and CD3, that specifically defines T/myeloid blasts. Moreover, we suggested that Notch3 directly controls the expression of these myeloid genes, by ChIP experiments on DP T cell samples, as well as transfection assay of activated Notch3 expressing vectors in both human and murine cell lines (kopkt1 and M31, respectively). Altogether, our data reveal an unexpected myeloid trait of the Notch-dependent T-ALL, that may have an important impact on the development of innovative multitargeted therapies.

Role of ζ-Crystallin in acidic tumor microenvironment

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Background. Metabolic reprogramming is one of the hallmark of cancer. In particular, most cancer cells rely on aerobic glycolysis, the well-known Warburg effect, which cause a chronic acidification of tumor microenvironment. In turn, acidosis induces a metabolic shift toward glutaminolysis and increase the apoptotic threshold of cancer cells affecting regulators of the apoptosis process belonging to Bcl-2 family.
Bcl-2 gene expression undergoes an intricate post-transcriptional regulation, mainly driven by interplay between an adenine-uracil rich element (ARE) in its mRNA and numerous ARE-binding proteins (AUBPs). Among the *Bcl-2* AUBPs, we identified ζ-Crystallin (CryZ) and highlighted its alteration in leukemia cells. Beside *Bcl-2*, CryZ is involved in post-transcriptional regulator of two major genes for glutaminolysis (*GLS* and *GDH*). Furthermore, in the effort to identify pharmacological inhibitors of CryZ, it has been demonstrated that aspirin-like analgesics are potent inhibitors of its enzymatic activity.
Hypothesis. On these bases, we hypothesized that CryZ is an important player in the survival pathways of cancer cells in acidic microenvironment and that aspirin could be a candidate pharmacological inhibitor of the binding of CryZ to its mRNA targets.
Results. We demonstrated that the extracellular acidity induced the expression of CryZ in leukemia Jurkat, cervical cancer HeLa, breast cancer MCF7, prostate cancer PC3 and melanoma A375 cell lines, and increased viability of Jurkat cells exposed to apoptotic stimuli. In addition, the *Bcl-2* mRNA stability was synergistically increased by both acidity and ectopically overexpression of CryZ in Hela cells. Finally, we preliminary demonstrated that aspirin reduced the binding of CryZ to *Bcl-2* mRNA in A375 cells.
Conclusions. Based on our data, we preliminary demonstrated that CryZ enhances *Bcl-2* expression in acidic microenvironment and emerges as an important pH-responsive element that confers a cytoprotective effect on cancer cells against apoptotic stimuli. In addition, the evidence that aspirin impairs CryZ binding to *Bcl-2* mRNA could lay the basis for development of innovative post-transcriptional therapeutic tools.

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Metabolomics analysis in non obese diabetic mice (NOD)

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Type 1 Diabetes (T1D) is an autoimmune disorder resulting from the selective destruction of insulin-producing β-cells in the pancreas. The diagnosis is preceded by a long prodromal period with progressive β-cell failure due to lymphocytes infiltration and by the appearance of islet specific autoantibodies [1]. The incidence of T1D has been steadily rising in developed countries. Although the exact etiology of T1D remains elusive, it is known that both genetic and environmental factors play a role in its immunopathogenesis. The non-obese diabetic (NOD) mouse represents the best animal model of human T1D [2]. Only a fraction of NOD mice do not progress to disease. Indeed, the incidence of spontaneous diabetes is 80 % in females and 20 % in males in our animal facility in Cagliari University (Monserrato). Nuclear magnetic resonance (NMR) spectroscopy based metabolomics is a powerful technique that can be used to characterize the metabolic profiles of biological fluids. Metabolome is sensitive to both genetic and early environmental factors influencing later susceptibility to chronic diseases. In this study NMR-based metabolomics analysis in conjunction with multivariate statistics was applied to examine changes in urinary mouse metabolites. We have analysed NOD mice and as controls C57BL/6, and DBA/1J strains with lower T1D proneness in order to highlight possible differences in metabolites profiles. A total of 15 females animals of 8 weeks of age were included in our study: 7 NOD, 3 C57BL/6 and 5 DBA/1J mice. Preliminary Partial Least Squares Discriminant Analysis (PLS-DA) model data, R2Y=0.85 and Q2=0.79, show a clear separation in three different metabolites patterns. The discriminant metabolites are lactate, citrate, hippurate, trimethylamine and taurine. Our data demonstrate that urinary metabolomics analysis may identify discriminating profiles indicating different metabolic pathways associated to animals having a different T1D proneness.

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Circulating sRAGE in the Diagnosis of Bone Metastasis

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Despite the clinical importance of bone metastasis, we still know little about their onset and progression. Currently the diagnostic tools are lacking of sensitivity and specificity, that were required for clear early diagnosis. Therefore we need to investigate the pathogenesis of bone metastatic invasion in order to improve diagnosis. The Wnt pathway has been describe as having an important role in bone carcinogenesis. sRAGE (the circulating form of the Receptor Advanced Glycosilated Endproducts), is involved in Wnt pathway [1] and has been recently describe to have a protective role in several inflammatory diseases, including neoplastic diseases. Being released in to circulation, serum sRAGE levels is measured as marker of disease status [2]. This study investigated the diagnostic potential of sRAGE to improve the detection and monitoring of bone metastasis. We measured sRAGE in a control group of healthy patients, in patients with primary tumors and in patients with bone metastasis. Serum sRAGE was also correlated with the Wnt inhibitors DKK-1 and Sclerostin, with the bone metabolism marker MMP-2, MMP-9 and TRAP5b and with the metastatic marker Survivin. sRAGE resulted significantly lower in primary tumor and metastatic patients compared to healthy patients. Moreover sRAGE displayed a strong negative correlation with DKK-1, Sclerostin, MMP-2, MMP-9, TRAP5b and Survivin. These results indicated that sRAGE can be considered a protective molecule in metastatic progression and it could have a diagnostic role in bone metastatic detection and monitoring.

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Dietary fructose as risk factor for neurodegenerative diseases: role of advanced glycation end-products

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Several studies indicate the involvement of advanced glycation end-products (AGEs) in neurodegenerative diseases. Recently, it has been evidenced that dietary fructose can evoke high levels of endogenous AGEs. Moreover, the rising consumption of fructose in industrialized countries has been related to cognitive impairment, but the impact of fructose-derived AGEs on hippocampus has never been investigated.
The present study aimed to evaluate in the hippocampus of C57Bl/6 mice fed a standard (SD) or a 60% fructose (HFRT) diet for 12 weeks the production of the most studied AGEs, carboxy methyllysine (CML), and related signaling pathways activation, focusing on the role of the glutathione-dependent enzyme glyoxalase (Glo-1), the main AGEs-detoxifying system.
In the hippocampus of HFRT mice an increased expression of the fructose receptor GLUT-5 and of its metabolizing enzyme ketohesokinase has been observed in glial cells and pyramidal neurons. HFRT diet evoked CML accumulation in the cell body of pyramidal neurons, followed by RAGE/NFkB signaling activation. A widespread reactive gliosis and altered mitochondrial respiratory complexes activity, paralleled by oxidative stress increase, have been evidenced in HFRT hippocampi. In addition, a translocation of Glo-1 from axons toward cell body of pyramidal neurons has been observed in HFRT mice, in relation to CML accumulation. Despite increased expression of dimeric Glo-1, its enzymatic activity was not upregulated in HFRT hippocampi, due to reduced glutathione availability, thus failing to prevent AGEs accumulation. The prevention of AGEs production by administration of the specific inhibitor pyridoxamine was able to prevent all the fructose-induced hippocampal alterations. In conclusion, a high fructose consumption, through AGEs accumulation, induces in the hippocampus the same molecular and metabolic alterations observed in early phases of neurodegenerative diseases, thus representing a risk factor for their onset.

High BCR-ABL/GUS^{IS} levels at diagnosis are associated with unfavorable responses to standard dose Imatinib

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Background. The approval of second-generation tyrosine kinase inhibitors (TKIs) for the first line treatment of Chronic Myeloid Leukemia (CML) has generated a need for early molecular parameters associated with inadequate responses to Imatinib Mesylate (IM).
Objective. We correlated quantitative determination of *BCR-ABL* transcripts at diagnosis with the outcome (defined according to the 2013 European Leukemia Net recommendations) of 272 newly diagnosed CML patients receiving IM 400 mg/die.
Methods. *BCR-ABL* transcripts were measured from peripheral blood samples drawn at diagnosis before patients received any pharmacological treatment using Real-Time Quantitative PCR (RQ-PCR). All molecular determinations were performed twice (in triplicates) on the same sample using either *ABL* or *glucuronidase-beta* (*GUS*) as reference genes. *BCR-ABL* values were then reported on the international scale (IS).
Results. With a median follow-up of 60 months, 65.4% of patients achieved an optimal response, 5.6% presented a response currently defined as "warning", 22.4% failed IM treatment and 6.6% switched to a different tyrosine kinase inhibitor because of intolerance to the drug. We recorded 19 deaths (6.9%), 7 (2.5%) attributable to disease progression. We applied Receiver Operating Characteristic (ROC) curves to define BCR-ABL/GUS^{IS} expression levels that would separate patients likely (i.e. below the threshold) or unlikely (i.e. above the threshold) to achieve multiple endpoints, namely: optimal response (OR), failure-free survival (FFS), event-free survival (EFS), transformation-free survival (TFS) and overall survival (OS). Employing the specific threshold calculated for each endpoint we found that high BCR-ABL/GUS^{IS} levels at diagnosis were associated with inferior probabilities of OR ($p<0.001$), FFS ($p<0.001$) and EFS ($p<0.001$). Elevated BCR-ABL/GUS^{IS} levels were also associated with higher rates of disease transformation to the accelerated phase or blast crisis ($p=0.029$) but not with OS ($p=0.132$).
Conclusions. High *BCR-ABL* transcripts at diagnosis measured by RQ-PCR employing *GUS* as a reference gene allow the identification of CML patients unlikely to benefit from standard dose IM that should be considered for alternative forms of treatment.

Metabolic reprogramming towards pentose phosphate pathway characterizes early stages of hepatocellular carcinoma and identifies a more aggressive subset

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A shift towards a Warburg metabolism in which aerobic glycolysis is increased has long been associated to neoplastic transformation. However, whether the switch from oxidative phosphorylation to glycolysis can occur at early stages of liver cancer development remains elusive. Using the Resistant-Hepatocyte model, we show that the acquisition of the Warburg phenotype is a very early event in rat HCC development as demonstrated by concomitant MCT4 expression and oxidation/inhibition of pyruvate kinase M2. We also observed inhibition of succinate dehydrogenase by the chaperone tumor necrosis factor receptor-associated protein 1 and an increase in the expression and activity of citrate synthase. In these preneoplastic lesions, metabolic reprogramming towards the Pentose Phosphate Pathway (PPP) was indicated by a strong increase in the expression and activity of glucose-6-phosphate dehydrogenase (G6PD). G6PD increased expression was observed exclusively in the highly proliferating KRT-19 positive preneoplastic lesions, considered the HCC precursor lesions in the R-H model, and was associated with low levels of miR-1, a miR targeting G6PD. Accordingly, forced expression of miR-1 down-regulated G6PD expression in HCC cells. Activation of the PPP has been suggested to be one of the mechanisms by which deregulated NRF2-KEAP1 signaling promotes cellular proliferation and tumorigenesis. Since in this rat model there is a sustained activation of the NRF2/KEAP1 pathway, we investigated the effect of NRF2 on G6PD and miR-1 expression. The results indicate that NRF2 silencing inhibits G6PD expression while increasing that of miR-1. Finally, an inverse correlation between miR-1 and its target genes involved in the PPP was found in human patients carrying HCC. Our results demonstrate that PPP activation is an early event in HCC development and that TRAP1 and NRF2 represent key regulators of metabolic reprogramming and ROS homeostasis in preneoplastic hepatocytes.

Evidence for a physiological role of Aβ in memory formation

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Background. Cyclic adenosine monophosphate (cAMP) regulates long-term potentiation (LTP) and ameliorates memory in healthy and diseased brains. Increasing evidence also shows that, under physiological conditions, low concentrations of amyloid β (Aβ) are necessary for LTP expression and memory formation. Based on these evidences, we tested the hypothesis of a functional correlation between cAMP, Aβ and LTP, in an attempt to reveal novel molecular mechanisms involved in memory formation.

Methods. In neuronal cultured cells and rat hippocampal slices, expression of the Aβ precursor protein (APP) was measured by RT-PCR and immunoblotting, whereas $A\beta_{42}$ was analyzed using specific ELISA. Electrophysiological LTP recordings were performed in hippocampal slices from wild-type and APP knockout mice.

Results. Our study shows, for the first time, that cAMP enhances LTP by stimulating the synthesis of APP and, in turn, the production of Aβ. In particular, our results indicate that PKA but not EPAC is involved in the cAMP-induced increase of APP and $A\beta_{42}$. Moreover, we demonstrate that cAMP requires translation, but not transcription, in order to increase APP and Aβ levels. Finally, we show that the reinforcing effects of cAMP on LTP are abolished in APP knockout mice, where Aβ cannot be produced, and are prevented in wild-type animals when the extracellular peptide is depleted by anti-Aβ antibodies.

Conclusions. The present data demonstrate that endogenous cAMP requires APP and Aβ to boost hippocampal LTP. Collectively, our study has revealed a novel cAMP/PKA/APP/Aβ molecular pathway through which the second messenger positively influences the cellular mechanisms of memory formation and adds further evidence for a physiological role of Aβ.

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The contribution of endogenous oxysterols in inducing intestinal tissue injury

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Oxysterols originate from cholesterol oxidation and are more reactive than unoxidized cholesterol. They have been involved in the progression of human diseases associated with inflammation, such as atherosclerosis and Alzheimer's disease, and recently considered in the pathogenesis of Inflammatory Bowel Disease (IBD). Oxysterols of dietary origin have been suggested to potentially interfere with mucosal intestinal epithelium homeostasis, by promoting and sustaining irreversible damage and inflammation.
However, implication of endogenous oxysterols in inducing intestinal damage has not yet elucidated. Endogenous oxysterols originate enzymatically, and most of them are considered as signaling molecules, being ligands of Liver X Receptors (LXRs) that function as master transcription factors in cell metabolism.
Increased expression and activity of matrix metalloproteinases (MMPs) may be implicated in intestinal injury in IBD, by inducing extracellular matrix (ECM) degradation and contributing to mucosal ulceration and inflammation.
Endogenous oxysterols have been hypothesized to lead to MMP-9 overexpression in unstable human atherosclerotic plaques, by causing excessive ECM proteolysis and plaque rupture. Therefore, we investigated the ability of principal endogenous oxysterols in inducing intestinal damage through the modulation of MMPs, and their inhibitors TIMPs, in differentiated CaCo-2 cells.
Among different oxysterols analyzed, 24- and 27-hydroxycholesterol significantly induced MMP-9/TIMP-1 and MMP-2/TIMP-2 imbalance. Furthermore, these oxysterols have been found to down- or up-regulate inflammatory cytokines expression in a dose-dependent manner, probably through LXR induction. However, the association oxysterols- LXR in ECM destabilization and inflammation needs clarification.
These preliminary results provide a new rationale for future studies on oxysterols-LXRs axis in modulating signaling pathways implicated in intestinal barrier destabilization and injury in IBD.

Investigating the potential of high density lipoproteins as anti-tumoral tool in prostate cancer

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Background. In Western countries prostate cancer (PC) is the most commonly diagnosed cancer in men. Androgen deprivation therapy is almost universal, but in some cases the progression to castration resistant PC (CRPC) occurs in 2-3 y. Reactive oxygen species (ROS) play an important role in malignant transformation, progression and aggressive phenotype of PC. Being the clinical management of CRPC a challenge and the prognosis poor, novel therapeutic agents are needed. High-density lipoprotein (HDL) particles are responsible for the reverse transport of cholesterol from peripheral tissues to the liver. Moreover, HDL shows anti-inflammatory and antioxidant properties which could negatively affect cell proliferation. Plasma-derived HDL are unsuitable for drug development because of safety concerns, large-scale production and heterogeneity. These limitations may be overcome by preparing synthetic HDL (sHDL), discoidal lipoprotein particles made with a purified apolipoprotein and a phospholipid.
Aim. To exert the anti-inflammatory and antioxidant properties of sHDL in human LNCaP (androgen-dependent phenotype) and PC3 (CRPC phenotype) cells, infering that such activities could negatively affect cell proliferation or increase cell sensitivity to classical cytotoxic agents.
Results. LNCaP were pulsed in 32.25 μM H2O2 for 1 h, then allowed to recover and proliferate for up to 72 h. H2O2 progressively enhanced LNCaP cell growth over the time-course experiment (24-72 h), reaching significance at 72 h (+ 27%, $p<0.01$). HDL (0.5 mg/mL) counteracted the H2O2-driven effect on LNCaP cell proliferation. Moreover, 1-h treatment with H2O2 (0.5 mM) significantly enhanced ROS production in LNCaP (+59%, $p<0.01$) and in PC-3 cells (+31%, $p<0.001$). In both cell lines the addition of HDL (0.5 mg/mL) was able to rescue the levels of intracellular ROS.
Conclusions. sHDL undergoing the clinical development phase may counteract the consequences of changes in ROS levels relevant to PC progression.

Pro-tumorigenic role of the CNBP-cMyc axis in colorectal carcinogenesis

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Colorectal cancer is the second leading cause of death from cancer among adult females and the third among males. The disease typically begins as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer.
c-Myc appears to play an important role in CRC pathogenesis and its overexpression is commonly observed in CRC samples, underscoring the importance of understanding how this oncogene is upregulated in CRC. The intracellular levels of c-Myc are subjected to a tight regulation from multiple factors. The CCHC-type Nuclear Binding protein (CNBP) appears to be one of these regulators. CNBP is a highly conserved zinc-finger protein that acts as nucleic acid chaperone and regulates transcription and translation. In our previous work we have demonstrated that CNBP promotes IRES dependent translation of Myc in *Drosophila melanogaster*, thereby regulating cell proliferation and developmental processes. Here we have investigated the conservation of this mechanism in mammals and its potential role in CRC tumorigenesis. We have found that CNBP binds the 5'UTR of cMyc *in vitro* and *in vivo* and promotes the IRES dependent translation of a cMyc bicitronic construct. We have observed that CNBP and cMyc are overexpressed in both mouse and human CRC samples analyzed, demonstrating the relevance of this mechanism in tumors. Notably, ablation of CNBP from CRC cells causes reduction of c-Myc protein and a consequent strong decrease of CRC growth *in vitro* and in xenografted animal models.
Collectively, these data illustrate a previously uncharacterized mechanism whereby, by promoting translation of cMyc, CNBP plays a key role in CRC pathogenesis. This data may have relevant implications in the diagnosis and treatment of CRC.

KCTD15, a new KCASH2 interactor and modulator of the Hedgehog pathway

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KCASH (KCTD containing, Cullin3 adaptor, suppressor of Hedgehog) family of proteins (KCASH1-3) has been recently identified as modulator of the Hedgehog (HH) signaling pathway. These proteins are characterized by a BTB domain required for the formation of a Cullin3 ubiquitin ligase complex and HDAC1 ubiquitination and degradation capability.
KCASH proteins play a critical role in cerebellar granule cells development and in Hedgehog-dependent medulloblastoma growth.
By performing a co-IP and mass spectrometry assay, we identified new KCASH2 interactors. The most represented is human KCTD15 (Potassium Channel Tetramerization Domain containing 15). KCTD15 belongs to a different subfamily of KCTD containing proteins. KCTD15 has been described to play a role in neural development, modulating the expression levels of Wnt and FGF during the formation of the neural plate border.
We present here our findings on KCTD15 contribution to the regulation of the Hh pathway.
We have verified that KCTD15 is indeed able to interact with KCASH2 both by *in vitro* GST pull down assays and by endogenous co-IP assay. This interaction is through a direct binding of the BTB/POZ domains present in both proteins.
KCTD15/KCASH2 interaction stabilizes the two proteins, increasing the efficiency of the Hh inhibition by KCASH2. We have also verified that KCTD15 inhibits the transcriptional activity of Gli1 by luciferase assay.
We demonstrate that, consistently with the pro-proliferative role of Hh on tumor cells, KCTD15 overexpression significantly reduces colony formation capability in human MB cells. These data suggest that KCTD15 modulates the Hh expression by the interaction with KCASH2.

Quercetin loaded into nanoparticles prevents neuroinflammation induced by oxysterols

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Chronic inflammation plays a fundamental role in Alzheimer's disease (AD)-related neuropathological changes, and results in neuronal dysfunction and death. Besides activation and proliferation of glial cells, recent evidence suggests that neurons can also react and contribute to neuroinflammation changes in the AD brain, by serving as sources of inflammatory mediators. Among risk factors for this neurodegenerative disease, a mechanistic link between altered cholesterol metabolism and AD has been suggested, and oxysterols (cholesterol oxidation products) appear to be the missing linkers between the two, because of their neurotoxic effects.
We observed that the oxysterols potentially implicated in AD pathogenesis, 24-hydroxycholesterol, 27-hydroxycholesterol, and 7β-hydroxycholesterol, induce the expression of some inflammatory mediators (CD36, β1-integrin, MCP-1, IL-8, MMP-9) in human neuroblastoma SH-SY5Y cells, via Toll-like receptor-4/cyclooxygenase-2/membrane bound prostaglandin E synthase. To confirm this evidence, cells were incubated with the flavonoid quercetin, a natural anti-inflammatory. Notably, its anti-inflammatory effects in SH-SY5Y cells were enhanced when it was loaded into β-cyclodextrin-dodecylcarbonate nanoparticles, versus cells pretreated with free quercetin
This drug delivery system might thus increase the neuroprotective effects of quercetin, by improving both its permeation across the blood brain barrier and its bioavailability to reach target cells, and it might be a new therapeutic strategy for preventing or reducing AD progression.

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