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Promoter methylation of TSC2 gene is involved TSC and LAM pathogenesis

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Tuberous sclerosis complex (TSC) and lymphangioleiomymatosis (LAM) are rare diseases. TSC is a genetic disease caused by mutation in *TSC1* or *TSC2* genes. TSC2 cells form hamartomas and might invade lungs causing the fatal diseases LAM. From an angiomylipoma (AML) of a TSC male patient we isolated a TSC2 smooth muscle (ASM) population. The growth of these cells was EGF-dependent (Lesma et al. 2005). They showed a constitutive S6 phosphorylation, and lacked tuberin as the result of TSC2 promoter methylation ($TSC2^{-/meth}$ ASM cells). Chromatin remodeling agents, such as trichostatin–A and 5-azacytidine de-methylated $TSC2^{-/meth}$ ASM cell promoter and induced tuberin expression. The blockade of EGF-receptor with specific antibodies results in cell death as shown in $TSC2^{-/-}$ ASM cells, a population previously isolated from an AML of female TSC patient. LAM cells migrate or metastasize to other organs; in fact cells with *TSC2* mutation have been found in AMLs and lung lesions of LAM patients (Henske et al. 2005). We isolated a population from chylous of a TSC/LAM patient, in which, as in $TSC2^{-/meth}$ cells, tuberin expression was induced by 5-azacytidine and trichostatin-A treatments. These cells required the supplementation of EGF for proliferation, such as $TSC2^{-/-}$ and $TSC2^{-/meth}$ ASM cells. Chylous TSC/LAM cells expressed marker for identification of mesenchimal characteristics, such as vimentin and SNAIL, while E-cadherin, usually not expressed in metastatized cancer cells, was not detectable. The acquisition of mesenchymal characteristics for cancer cells is a transient event that might be important for migration and tissue invasion (Thiery et al. 2009). Chylous TSC/LAM cells switched from a short floating stage, with low S6 phosphorylation levels, to a stage of adhesion with high S6 phosphorylation. These data suggest a novel therapeutic approach for the control of TSC and LAM abnormal cell growth using anti-EGFR antibody in addition to rapamycin, and, in some cases, the chromatin remodelling agents. Moreover, our data confirm the evidence that TSC pathogenesis might originate from an epigenetic defect.

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Obestatin affords cardioprotection to the ischemic/reperfused isolated rat heart and inhibits apoptosis in cultures of similarly stressed cardiomyocytes

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Obestatin, a newly discovered peptide encoded by the ghrelin gene, induces expression of genes regulating pancreatic β-cell differentiation, insulin biosynthesis, and glucose metabolism. It also activates anti-apoptotic signalling pathways such as PI3K and ERK1/2 in pancreatic β-cells and human islets. Since these kinases have been shown to protect against myocardial injury, we sought to investigate whether obestatin would exert cardioprotective effects. Both isolated perfused rat heart and cultured cardiomyocyte models of ischemia/reperfusion (I/R) were used, measuring infarct size and cell apoptosis as end points of injury. The presence of specific obestatin receptors on cardiac cells, as well as the signalling pathways underlying the obestatin effect was also studied. In isolated heart, the addition before ischemia of rat obestatin-(1-23) reduced infarct size and contractile dysfunction in a concentration-dependent manner, whereas obestatin-(23-1), a synthetic analogue with inverse amino acid sequence, was ineffective. The cardioprotective effect of obestatin-(1-23) was observed at concentrations of 10-50 nM and was abolished by inhibiting PI3K or PKC by addition of wortmannin (100 nM) or chelerythrine (5 μM), respectively. In rat H9c2 cardiac cells or isolated ventricular myocytes subjected to I/R, 50 nM obestatin-(1-23) reduced cardiomyocyte apoptosis and reduced caspase-3 activation; the anti-apoptotic effect was blocked by inhibition of PKC, PI3K or ERK1/2 pathways. In keeping with these functional findings, radioreceptor binding results revealed the presence of specific high-affinity obestatin binding sites, mainly localized on membranes of ventricular myocardium and cardiomyocytes. Our data suggest that, acting on specific receptors, obestatin-(1-23) activates PI3K, PKCε, PKCδ, and ERK1/2 signalling and protects cardiac cells against myocardial injury and apoptosis induced by I/R.

Role of autologous rabbit adipose-derived stem cells in the repairing process of critical bone defect

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One of the most important issues in orthopaedic surgery is the loss of bone resulting from trauma, infections, tumours or congenital deficiencies (Parikh 2002). Nowadays, an innovative approach to solve this problem includes tissue engineering techniques in which the use of autologous cells and bio-degradable or bio-absorbable scaffolds with adequate mechanical properties and interconnecting pores is required to allow cellular infiltration, graft integration and vascularisation (Cancedda et al. 2007; Jager et al. 2009). We believe that adipose-derived stem cells (ASCs), with their great availability and strong osteogenic potential, may represent, alone or in combination with osteoconductive scaffolds, an efficient approach for bone regeneration.
Since we wanted to evaluate the ability of ASCs to regenerate osteochondral defects in pre-clinical models, we have characterized ASCs isolated from rabbit (rbASCs) and we have shown their ability to maintain an undifferentiated state and to efficiently differentiate towards osteogenic lineage, alone or with biomaterials (Arrigoni et al. 2009).
In this study, rbASCs were isolated from interscapular adipose tissue, expanded in vitro and reimplanted in a full-thickness bone defect in the proximal epiphysis of tibia of twelve New Zealand rabbits as follows: sham (just defect), insertion of hydroxyapatite disk (HA), of ASCs alone (ASCs), and of hydroxyapatite-ASCs seeded-disk (HA-ASCs; de Girolamo et al. 2010). Each ASCs population was tested *in vitro*: all of them show a high proliferation rate with a doubling time of 56.5±16.9 h, a marked clonogenic ability (3.0±1.6%) and osteogenic differentiation potential evaluated by extracellular calcified matrix deposition (161.6±77.9% increase of osteo vs undifferentiated ASCs). We have also observed a significant increase in osteonectin, osteocalcin, and collagene type I protein expression by Western blot analyses. Collagen production is also confirmed by collagen type I production on osteogenic differentiated rbASCs cultured in the absence or in the presence of hydroxyapatite granules (105.0±61.9% on monolayer and 35.8±12.3% on HA). Eight weeks after surgery, gross appearance, X-ray, bone mineral density (BMD) and histological/immunohistochemical analyses were performed.
The macroscopic analyses of the tibias show satisfactory filling of the lesion without any significant differences in term of stiffness between groups treated with or without cells ($P>0.05$). In both the scaffold-treated groups, a good osteointegration was radiographically observed. Even if HA was not completely reabsorbed, ASCs-loaded HA displayed a more efficient scaffold resorption than the unloaded ones (Wakitani et al. 1994). Furthermore, histological analyses show the osteogenic abilities of the scaffold-treated defects with respect to the scaffold cell-free samples, and in particular, the new formed bone was more mature and similar to native bone in presence of ASCs. These data were also confirmed by immunohistochemical analyses evaluating the positive cells (osteoblast and osteocyte) and new matrix bone formation with two important osteogenic markers osteopontin and collagen type I.
These results indicate that autologous ASCs-hydroxyapatite construct is a potential treatment for the regeneration of bone defects. Moreover, since scaffold loaded with ASCs produced a more mature healing tissue with a more abundant presence of osteoblasts with respect to the empty scaffolds, this approach may be useful in a hypothetical one-step bone defect treatment reducing the rehabilitation’s time in the clinical pratice.

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Pharmacological modulation of AMP-activated protein kinase as a novel strategy for the treatment of inflammatory bowel diseases

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Inflammatory bowel diseases (IBDs) are severe chronic pathologies characterized by a wide range of gastrointestinal and extra-digestive symptoms. The identification of novel drugs for treatment of IBDs represents an area of interest and active investigation, since currently available therapeutic options do not ensure adequate improvement/remission of the disease or induce severe adverse reactions in a significant proportion of patients (Antonioli et al. 2008). Acadesine (ACA), an activator of AMP-activated protein kinase (AMPK), has been shown to act in a site- and event-specific manner against inflammation (Drew et al. 2008) through the local increment of endogenous adenosine levels (Zhang et al. 2006). However the effects of ACA on intestinal inflammation have not been investigated. The aims of this study were to examine the effects of ACA in an experimental model of colitis in rats, and to evaluate the underlying anti-inflammatory mechanisms. The effects of ACA or dexamethasone (DEX, used as a standard anti-inflammatory drug comparator) were tested in male Sprague-Dawley rats (n=6 for each group) with colitis induced by intrarectal administration of 2,4-dinitrobenzenesulfonic acid (DNBS, 15 mg/rat), to assess systemic (body and spleen weights) and tissue inflammatory parameters [macroscopic and microscopic damage, tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), superoxide anion production (dihydroethidium fluorescence) and malondialdehyde (MDA) levels]. Animals received ACA (1, 3, 10 or 30 mg/kg/day), DEX (0.1 mg/kg/day) or vehicle intraperitoneally for 6 days, starting 1 day before DNBS administration. In order to characterize the mechanisms through which ACA ameliorated intestinal inflammation, a subset of experiments were performed in rats with colitis treated with compound C (a selective AMPK inhibitor) and 8-(4-sulfophenyl)theophylline (8-SPT, a non-selective adenosine receptor antagonist), given either alone or in combination with ACA. Colitis was associated with a decreased body weight (-10±5 g vs vehicle; $P<0.05$) and increased spleen weight (+22±3% vs vehicle; $P<0.05$). Microscopic damage score, tissue TNF-α and oxidative stress were also increased, while tissue IL-10 levels were markedly reduced. Treatment with ACA, but not DEX, improved body weight. Both drugs counteracted the increase in spleen weight and ameliorated the histological damage (DNBS: 4.8±0.9; ACA 10 mg/kg/day: 3.1±0.7; DEX: 2.0±0.4; $P<0.05$ vs DNBS; ANOVA). A reduction of TNF-α and increase in IL-10 tissue levels were also recorded in rats treated with test drugs. Moreover, ACA or DEX ameliorated colonic oxidative damage. Preliminary data showed that treatment with compound C or 8-SPT did not worsened DNBS-induced colitis. However, when administered together both drugs were able to counteract the beneficial effects exerted by ACA on colonic inflammation. Overall, the present results suggest that the AMPK activator ACA counteracts bowel inflammation, acting through a rearrangement of pro-inflammatory/anti-inflammatory cytokine balance and a reduction of oxidative stress. The beneficial effects of ACA on bowel inflammation depend on the increase in endogenous adenosine availability. These findings suggest that the pharmacological modulation of AMPK function could represent a promising strategy to develop novel classes of drugs effective against intestinal inflammation.

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Nociceptin/orphanin system, α-synuclein, and parkin gene expression in animal models of Parkinson's disease

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Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder of movement of likely multi-factorial origin. A significant genetic element in the aetiology of PD is suggested (Gasser 1998) and, in addition, environmental toxins such 6-OHDA (Ungerstedt 1968) and MPTP (Langston and Ballard 1983) as well as agricultural chemicals like rotenone, paraquat (PQ) and maneb (MB) (Gorell et al. 1998, Menegon et al. 1998) have been associated with PD. The aim of this study is to investigate the possible role of nociceptin/orphanin FQ peptide (N/OFQ-NOP) system in the pathogenesis of the disease in an animal model developed using PQ and/or MB. Weight, locomotion activity and specific behaviours of the animals have been registered daily. We determined the levels of tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine (DA) synthesis, in the substantia nigra (SN), using Western analysis. Quantification of α-synuclein, parkin, and nociceptin system genes expression in SN of all animals treated was performed by RT-PCR techniques. Adult male Sprague-Dawley rats were used, injected i.p. twice a week for 4 weeks (total of 8 injections) with saline solution (vehicle, n=10), PQ (10 mg/kg, n=10), MB (30 mg/kg, n=10), PQ+MB (10+30 mg/kg, n=10) and PQ+MB (5+15 mg/kg, n=10). Doses have been chosen based on previously reports (Thiruchelvam et al. 2000; Cicchetti et al. 2005). All treated animals began to lose weight and several animals died after the 3rd injections of PQ+MB high dose (10+30 mg/kg) and after the 5th injection of PQ (10 mg/kg) showing weight decrease and clear signs of toxicity. The locomotion activity and the animal welfare decrease evidently but not significantly in treated animals compared with control group. A decrease of TH immunoreactivity was observed in animals treated with PQ+MB (5+15 mg/kg) confirming the dopaminergic damage. The N/OFQ did not show statistically significant differences toward control group, but it was possible to observe that gene expression levels of NOP receptor raised significantly, allowing us to hypothesize the development of a behaviour of a preclinic stadio. Interestingly, we found a marked increase and a slight decrease in α-synuclein and parkin gene expression, respectively. These data are consistent with the pathology, thus indicating a deposit of these proteins and an opposite action between them (Baptista et al. 2004).

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Free radical scavenging activity from *Casimiroa* spp. extracts

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Free radicals, particularly reactive oxygen species (ROS), are involved in the pathogenesis of several chronical and degenerative diseases. This study was designed to investigate the antioxidative properties of natural extracts and isolated compounds of *Casimiroa edulis* and *Casimiroa pubescens* Llave et Lex (Rutaceae).

The genus *Casimiroa* is widely spread in the tropical and subtropical areas of Central America: the decoction of leaves and seeds, together with the consumption of the edible fruits are quite diffused as folk remedies to relieve hypertensive ailments, insomnia, rheumatisms, and arthritic pains. To the best of our knowledge, there are no available scientific data directly related to the benefit of scavenging activity toward radical-mediated disease prevention, such as for coronary heart disease, cancer, Alzheimer, arthritis, and diabetes as well as degenerative processes associated with aging (Halliwell et al. 1992), in consequence of membrane damage, protein modification, enzyme inactivation, and DNA damage induced by free radicals.

The antioxidant effect was evaluated by α,α-diphenyl-β-picrylhydrazyl (DPPH·) decolorization, according to the method reported by Brand-Williams et al. (1995); the assay was performed first with TLC plate as positive antioxidant control and then with a spectrophotometric analysis to measure the effective *in vitro* scavenging activity. We used N-acetyl-L-cysteine, pyrogallic acid, α-tocopherol, L-ascorbic acid, and quercetin as antioxidant standards. *Casimiroa edulis* hexanic leaf extract (Ce5), seed extract (Ce7), and methanolic seed extract (Ce8) at 600 μg/ml showed an antiradical activity of 77.0±9.2%, 24.2±2.9%, and 72.0±5.6%, respectively. Also *C. pubescens* methanolic leaf extract (Cp10) and seed extract (Cp12) at the same concentrations pointed out an antioxidant activity of 84.7±0.2% and 58.4±4.3%, respectively.

According to the phytochemical composition of the extracts, the DPPH· screening was performed also with several organic compounds specifically identified in the genus, such as imperatorin, herniarin, 7-methoxy-3-(2-methylbut-3-en-2-yl)cumarin, and pentamethoxyflavone. Imperatorin and pentamethoxyflavone (5 mM) revealed an antioxidant activity of 71.3±0.2% and 30.7±1.3%, respectively, while the cumarin derivatives showed a very low antioxidant profile.

Experimental evidences indicated a non-linear relationship between the antioxidant concentration and the DPPH· scavenging activity: as a consequence, the measurement of the EC_{50} seemed quite problematic. For this reason we used a mathematical software to process the data and then obtain a relation between antioxidant activity and sample concentration (Locatelli et al. 2009).

Hence, *Casimiroa* extracts and some isolated constituents - mainly imperatorin and pentamethoxyflavone - have shown a remarkable radical scavenging activity; research is progressing to characterize their effects on LDL oxidation.

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Drug related-gene polymorphisms could influence the survival in esophageal cancer patients receiving neo-adjuvant radio-chemotherapy

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The inter-individual variability in the efficacy of anticancer chemotherapy may derive, at least in part, from variants (polymorphisms) of genes, whose expression's products play a fundamental role in drug response. In particular, the genes of interest are those involved in the metabolism and in the transport of drugs, and those codifying for enzymes that are target of therapy (Wu et al. 2005). The aim of the work was to evaluate the influence of gene polymorphisms in relation to the drug response efficacy, with regard to survival (event free and overall) in patients affected by locally advanced esophageal cancer, treated with a neo-adjuvant schedule including weekly docetaxel (35 mg/mq) and cisplatin (25 mg/mq), protracted venous infusion of 5-fluorouracil (150 mg/mq/die) and concomitant radiotherapy for 8 weeks followed by surgery.
The following genes were considered: thymidylate synthase (TYMS), as 5-fluorouracil target; methylenetetrahydrofolate reductase (MTHFR), whose function is a limiting factor for the inhibition of TYMS (Langevin et al. 2009); glutathione S-transferaseP1 (GSTP), mainly involved in the conjugation and inactivation of platinum derivatives (Li et al. 2010); P-glycoprotein (MDR-1), drug-resistance associated efflux pump of hydrophobic anticancer drugs including taxanes (Wu et al. 2006); XPD, XRCC1, ERCC1, XRCC3, and XPA, as genes implicated in DNA repair (Liu et al. 2007; Tse et al. 2008; Font et al. 2008; Guo et al. 2008;) and myeloperoxidase (MPO), as a representative gene involved in the immune system response (Matsuo et al. 2001).
One hundred-three patients (87 male, 16 female) were enrolled, aged 60±7 years old. Median follow-up was 25 months. Genomic DNA was extracted from peripheral blood lymphocytes and MTHFR C677T, MTHFR A1298T, TYMS VNTR 2R/3R, TYMS 3'UTR ins-del, GSTP1 Ile^{105}Val, GSTP1 Ala^{114}Val, MDR1 G2677T, MDR1 C3435T, XRCC1 Arg^{399}Gln, ERCC1 C118T, XRCC3 Thr^{241}Met, XPD A751C, XPA A23G, and MPO G463A were genotyped through RFLP analysis. Associations between gene polymorphisms and survival were analysed through Log rank test.
The event free (EFS) and overall (OS) median survival times have been reached. Up to now 37 patients suffered a disease relapse while 31 died. Variant distribution of all the genes was in Hardy-Weinberg equilibrium.
The MDR2677 GT genotype had a significant worse EFS (P value=0.0138) compared to MDR2677 GG and GT genotypes. There was no significant association between MDR G2677T polymorphism with OS. About the MDR C3435T polymorphism not significant association was found with clinical outcome.
The XPA23 AA genotype showed a significant increase risk of relapse (P value=0.04) and death (P value=0.0189) when compared with the other genotype AG and GG.
For the other genetic variants the analysis did not reveal any significant association with EFS and/or OS.
The analysis was extended to the valuation of possible associations between gene polymorphisms and pathological response but it did not get any significant result.
Our results in MDR2677 and XPA polymorphisms support the hypothesis that genetic variants could be useful to determine individuals with reduced survival following esophageal cancer chemotherapy and to identify some parameters for a customized therapy.
These findings need to be confirmed and clarified in further analysis, for example by a multivariate analysis that could compare more gene variants at the same time.

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Protective effects of melanocortins on short-term changes in a rat model of traumatic brain injury

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A treatment for traumatic brain injury (TBI) remains elusive despite compelling evidence from animal models for a variety of therapeutic targets. Numerous animal models have been developed to address the wide spectrum of mechanisms involved in the progression of secondary injury after TBI (Marmarou et al. 1994). To further compromise the patient's prognosis is the lack of clinically effective treatments to assist recovery from injury. Those individuals that survive TBI continue to live for many years with disabilities conferring large emotional and financial burdens. We used a rat model of diffuse TBI, the impact-acceleration model (O'Connor et al. 2007). In this study we investigated the molecular and histological changes induced by TBI and the possible protective effects of melanocortins (Ottani et al. 2010; Giuliani et al. 2009). Brain tissue NO synthesis (by Griess reaction), phosphorylation level of the two protein kinases ERK 1/2 and JNK, TNF-alpha expression (by Western blot), and brain histological damage were evaluated 24 and 48 h after insult. Post-traumatic administration of melanocortin (3 and 6 h after injury) reduced TBI-induced upregulation of ERK and JNK phosphorylation, and TNF-alpha expression. These molecular changes were associated with a reduction in brain NO synthesis at both time points. These results were in agreement with a reduced brain tissue damage as highlighted by histopathological findings. The findings of our study clearly indicate that the anti-inflammatory effect of melanocortins could be useful for the treatment of diffuse TBI.

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The oxidative stress drives fibroblast growth factor release in endothelial cells

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Disturbances in normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.
The oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to readily detoxify the reactive intermediates or easily repair the resulting damage. Many pathological conditions are associated with ROS production as metabolic syndrome, ischemia/reperfusion, inflammation, and neurodegenerative diseases.
Fibroblast growth factor 2 (FGF-2), a member of the fibroblast growth factor family, is a multifunctional protein involved in physiological and pathological processes such as angiogenesis, wound healing, and tissue repair. Different molecular isoforms of FGF-2 have been characterized and only the 18 kDa isoform is secreted by unconventional secretion (Presta et al. 2005).
FGF-2 has a high affinity for cell surface heparan sulphate proteoglycans (HSPG), where it is stored and which participate in the formation of stable and active FGF-2/FGFR complexes.
The aim of this work was to analyze the role of oxidative stress on FGF-2 release and signalling in microvascular endothelium.
Coronary venular bovine endothelial cells (CVEC) were treated for 24 h with 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), a redox-cycling agent known to induce intracellular superoxide anion formation. DMNQ induced a significant decrease of FGF-2 expression analyzed by Western blotting. Since the oxidative stress was not involved in FGF-2 mRNA transcription, the conditioned medium of CVEC was collected, clarified by centrifugation and lyophilized, and analyzed by Western blotting and ELISA for FGF-2 release. We found that the oxidative stress increased FGF-2 release in the conditioned medium.
Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition and degradation and MMP2 has been shown to release FGF-2 (Finetti et al. 2008). We thus analyzed the medium by zymography for the detection of MMP2 enzyme activity and by Western blotting for MMP2 expression. In the cells treated with DMNQ, MMP2 expression and activity increased. To confirm the relation FGF-2/MMP2, CVECs were treated with MMP2 inhibitor and the conditioned medium was analyzed by ELISA for FGF-2 quantification. Surprisingly the MMP2 inhibitor did not interfere with the FGF-2 release.
Since FGF-2 release is mediated by shedding of membrane vesicles from cells (Taverna et al. 2003), the conditioned medium from CVECs was collected and ultracentrifuged to precipitate potential microvesicle-bound proteins (pellet). The pellets were again analyzed by Western blotting for FGF-2 expression. In CVEC treated with DMNQ a significant increase of vesicle containing FGF-2 was found.
The caspase-1 activity is an important regulator of unconventional protein secretion. It is know that its activity is mediated by a cytosolic molecular complex, the "inflammasome" (Keller et al. 2008). In CVEC, DMNQ significantly reduced the intracellular caspase-1 expression, while increased the release of FGF-2/caspase-1 complex analyzed by immunoprecipitation.
In conclusion, the oxidative stress decreased intracellular FGF-2 in microvascular endothelial cells and increased the FGF-2 release on extracellular space. In this process the MMP2 activity seems not involved, rather the FGF-2 release could be mediated by FGF-2/caspase-1 complex formation.

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Role of prostanoids in inflammatory bowel diseases: new insights arising from microsomal prostaglandin E synthase-1 knockout mice

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Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), represent a chronic, relapsing and remitting inflammatory condition that affects individuals throughout life. Nonsteroidal anti-inflammatory drugs (NSAIDs), acting by the reduction of prostanoid generation (FitzGerald et al. 2001), have been associated with worsening of IBDs (Kefalakes et al. 2009). Among various prostanoids affected by NSAIDs, prostaglandin (PG)E_2 has received much attention, but its specific role in IBDs remains unclear. Constitutive PGE_2 production in the intestine appears to have a protective effect on the integrity of the epithelial intestinal wall, while enhanced PGE_2 generation in inflammatory conditions could contribute to the perpetuation of inflammation (Dey et al. 2006). PGE_2 biosynthesis is the result of the coordinate action of cyclooxygenase (COX) and PGE synthases. In particular, microsomal PGE synthase-1 (mPGES-1) is an induced isoform, functionally coupled with COX-2, shown to be an important source of inflammatory PGE_2 (Murakami et al. 2000). The aim of this study was to clarify the role of PGE_2 in IBDs by using mPGES-1-deficient mice. In particular, in an experimental model of IBDs, mPGES-1 wild type and null mice have been used to assess the impact of mPGES-1 genetic deletion on: i) disease progression; ii) the generation of PGE_2 and other prostanoids *in vivo*.

Acute colitis was induced in mPGES-1 wild type and null mice by 2% dextran sulfate sodium (DSS) in drinking water for 5 days. Disease progression was determined at crucial time points (day 0, 4, 5, and 6) by assessment of disease activity index (DAI), which was calculated as the sum of separate scores, evaluating blood in stool (EMO SCORE) and stool consistency (STOOL SCORE) (Buanne et al. 2007). In 24-h urine collected both in basal condition (before DSS administration) and on day 5 of DSS treatment, 11alpha-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M), 2,3-dinor-6-keto PGF1alpha (PGI-M), 2,3-dinor- thromboxane B_2 (TX-M) and 11,15-dioxo-9alpha-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (PGD-M), as indices of PGE_2, prostacyclin (PGI_2), TXB_2 and PGD_2 biosynthesis *in vivo*, respectively, was assessed by liquid chromatography-mass spectrometry, as previously described (Wang et al. 2008).

During DSS administration at day 4, a worsening of the clinical symptoms of the disease was observed in mPGES-1 null mice vs wild type mice. In particular, the presence of faecal blood was significantly increased [EMO score (mean±SEM, n=6): 2.7±0.3 vs 1.3±0.5, respectively, $P<0.05$]. However, at day 5, a further progression of the disease was observed for mPGES-1 null mice and wild type mice, both showing the maximal DAI score value (DAI score: 5.0±0.5 vs 5.0±0.4, respectively). In mPGES-1 null mice, urinary excretion of PGE-M was significantly lower than in wild type mice, both in basal conditions (9.8±1.6 vs 18.5±2.3 ng/mg creatinine, respectively; P=0.02) and in DSS-induced acute inflammation (7.6±0.3 vs 31.3±7.4 ng/mg creatinine, respectively; P=0.04). Interestingly, urinary excretion of TX-M was significantly higher in mPGES-1 null mice than in wild type mice both in basal conditions (332.9±10.0 vs 233.6±21.5 ng/mg creatinine, respectively; P=0.007) and in DSS-induced acute inflammation (527.1±38.5 vs 291.0±36.04 ng/mg creatinine, respectively), while PGI-M and PGD-M levels were not significantly affected by mPGES-1 genetic deletion. In summary, these results show that in the acute phase of DSS-induced colitis, reduction of PGE_2 generation by mPGES-1 genetic deletion was associated with enhanced TXB_2 generation vs wild-type. These biochemical changes translated into a faster appearance of clinical symptoms of the disease, in particular of faecal blood. In conclusion, the present study supports a protective role of PGE_2 during the acute phase of intestinal inflammation. The possible contribution of thromboxane in the development of DSS-induced colitis requires further investigation.

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Possible role of hydrogen sulphide (H_2S) in the antihypertensive effect of zofenopril

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Hydrogen sulphide (H_2S) is an endogenous gas produced from L-cysteine by two different enzymes: cystathionine-β-synthase (CBS) and cystathionine γ-lyase (CSE). Both enzymes are pyridoxal-5'-phosphate-dependent and are widely distributed in mammalian tissues, in particular CBS is predominantly expressed in central nervous system whereas CSE is highly localized in the vasculature (Bucci et al. 2009; Yap et al. 2000). In recent years, accumulating evidences have suggested that H_2S is a newly modulator in vascular homeostasis both *in vivo* (Zhao et al. 2003) and *in vitro* (Hosoki et al. 1997); moreover it has been observed that alterations on H_2S biosynthesis is implicated in different vascular pathologies as: diabetes (Brancaleone et al. 2008), septic shock (Mok et al. 2004), and hypertension (Yan et al. 2004). Angiotensin converting enzyme (ACE) inhibitors are widely used as therapeutics, and their ability in controlling hypertension and risk of cardiovascular death is well established. However, it has been shown that ACE-inhibitors, beyond their classical action on renin-angiotensin system, exert a protective action on cardiovascular system. In fact they: improve endothelial function, cardiac and vascular remodelling; reduce atherosclerosis (de Nigris et al. 2001) and vascular reactivity in a experimental model of diabetes (Bucci et al. 2008). Among all ACE inhibitors, it has been demonstrated that zofenopril have a better protective action than enalapril (E) in different cardiovascular pathologies (Frascarelli et al. 2004). Therefore aim of this study was to evaluate if the better action of zofenopril compared to E, could be linked to the sulphydrilic group in the molecular structure of its active metabolite zofenoprilat (Z). The study was conducted *in vivo* measuring systemic blood pressure (SBP) in conscious animals by using the standard tail-cuff method in spontaneous hypertensive rats (SHR) vs Wistar Kyoto (WKY) as control strain. Different groups of animals of both strains were treated with equimolar doses of Z (10 mg/kg/die), E (6 mg/kg/die) or vehicle for two weeks. Pharmacological treatments started when the SBP of SHR rats was significantly higher than SBP of WKY (218.7±6.8 mmHg vs 143.2±3.3 mmHg, n=10, $P<0.001$ SHR and WKY, respectively). Both Z and E treatments significantly reduced SBP in SHR rats (155.4±4.3 mmHg and 172.8±4.6 mmHg vs 218.7±6.8 mmHg, n=10, $P<0.001$, Z vs vehicle; $P<0.001$ E vs vehicle; Z, E and vehicle, respectively). However, the antihypertensive effect of Z was significantly greater than the antihypertensive effect of E treatment (155.4±4.3 mmHg vs 172.8±4.6 mmHg, n=10, $P<0.05$, Z vs E). Moreover in WKY rats only Z treatment significantly reduced SBP (110.5±4.5 mmHg vs 143.2±3.3 mmHg, n=10, $P<0.001$, Z and vehicle, respectively). In order to evaluate the involvement of sulphydrilic group in Z antihypertensive action, we have measured H_2S levels in plasma of SHR and WKY with different treatments. In blood samples of SHR there was a significant reduction of H_2S compared to the levels observed in WKY (9.8±1.0 vs 17.3±2.5 nmols/mg protein/min, $P<0.05$; SHR and WKY, respectively). In SHR rats, Z treatment restored H_2S levels to control values (16.2±1.4 vs 11.2±1.5 nmols/mg protein/min, $P<0.05$; Z and SHR, respectively) meanwhile E treatment did not modify the levels of H_2S. These data suggest that the major antihypertensive effect of Z compared to E both in SHR and in WKY strains could be imputable to the sulphydrilic group presents on Z chemical structure.

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Resveratrol treatment: functional assay for drug effectiveness in Duchenne muscular dystrophy by using the *in vivo* model of exercised mdx mice

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Duchenne muscular dystrophy (DMD) is the most common and wasting form of muscular dystrophy due to an X-chromosome mutation leading to the absence of dystrophin in skeletal muscle (Hoffman et al. 2001). A similar biochemical defect also occurs in the mdx mouse, the most widely used animal model of DMD. The absence of this protein leads to sarcolemma weakness; consequently the stress of contraction triggers a complex series of events finally leading to muscle fibre degeneration. An important role in this process can be played by the alteration of calcium homeostasis, also sustained by an abnormal activity of voltage-independent calcium channel population (Fraysse et al. 2004; Rolland et al. 2006). In parallel, reactive oxygen species (ROS), either released by neutrophils or produced *in situ* by cytokine signalling, calcium and/or functional ischemia, may cause oxidative stress with sarcolemmal damage and leakiness. A growing number of evidence supports the potential link between mechanical challenge, alteration of calcium homeostasis and ROS-induced oxidative stress as well as their role in triggering and sustaining muscle degeneration and dysfunction. Possible therapeutic strategies may use drugs able to target pathogenetic events. Increasing evidences show that sirtuin1 (Sirt1), a known NAD+ dependent deacetylase modulates metabolic functions and improves reaction to stressors and longevity. Part of these actions are also mediated by the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), a key modulator of muscle metabolism (Cantó et al. 2009). PGC-1α overexpression in dystrophic mdx mice leads to milder signs of pathology and an improved function both in normal condition and after intense physical exercise (Handschin et al. 2007). Then, drugs able to activate Sirt-1/PGC-1α pathway may have positive effects in muscular dystrophy. We performed a proof-of-concept study by evaluating in treadmill-exercised mdx mice the effects of a chronic treatment with resveratrol (100 mg/kg; 6 days/week i.p. for 4-6 weeks), a known Sirt-1 activator, in comparison with those of a similar treatment with α-methyl-prednisolone (PDN 1 mg/kg i.p). *In vivo*, resveratrol and PDN similarly counteracted the exercise-induced decrease of maximal and normalized fore limb strength, while a partial amelioration of resistance to exercise was observed. *Ex vivo*, similarly to PDN, resveratrol treatment slightly ameliorated mechanical threshold, an electrophysiological index of calcium homeostasis, but did not exert any significant effect on isometric twitch and tetanic tension of EDL muscle. However, in contrast with PDN, a significant reduction of plasma creatine kinase and lactate dehydrogenase (58% and 78%, respectively) was observed in resveratrol-treated animals. Also, resveratrol and PDN produced about 70% reduction of fibres positive to dihydroethidium, a marker of superoxide anion production, in *tibialis anterior* muscle. An improvement of histology profile was observed in *gastrocnemious* muscle, along with a slight decrease of NF-κB positive fibres. In addition, resveratrol > PDN increased the expression of utrophin, a surrogate analogue of dystrophin, as evidenced by immunofluorescence assays. In conclusion, the results suggest that resveratrol may exert protective effect in dystrophic muscles, likely by enhancing metabolic pathways that contrast oxidative stress and reinforcing sarcolemma by utrophin overexpression.

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Different alcohol exposures induce selective alterations on the expression of the endogenous opioid system genes : possible epigenetic mechanisms

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Alcohol is the most socially-accepted addictive drug and alcoholism is the leading cause of disability and deaths. Many evidences suggest that ethanol alters neural activity through interaction with endogenous opiod systems (Koob et al. 1998). In both humans and animals, chronic ethanol exposure induces alterations in peptide level in brain tissues (Gianoulakis et al. 1996), in receptor densities, and induces a modifications of the mRNA coding for both peptides and receptors (Cowen et al. 2001). The endogenous opioid system seems to play a key role in alcohol actions and considerable neurological evidence indicates that the prefrontal cortex mediates “executive” functions including behavioral autonomy and self-control.
The aim of the study is to investigate the molecular mechanisms of adaptive transformations occurring at the cellular level in brain opioid systems after alcohol exposure.
Sprague Dawley rats received intragastric administration resembling human drinking with several hours of alcohol exposures: water or 1.5 g/kg alcohol (20% in water) three times daily for 1 (acute) or 5 (chronic) days. Brains were dissected 30 min (acute and chronic/dependent groups), 20 h (early withdrawal) or 3 days (late withdrawal) after the last administration. Real-time RT-PCR was used to assess mRNAs of interest abundances and amounts of specific immunoprecipitated DNA fragments at genes promoter.
Prodynorphin gene expression resulted up-regulated following 1 day of ethanol, in both amygdala and prefrontal cortex and, in dependent animals just in the amygdala, whereas a down-regulation was observed in the prefrontal cortex. Pronociceptin mRNA was increased in the amygdala of acute, dependent and early withdrawal groups. A potential epigenetic mechanism of gene regulation was observed in the amygdala in prodynorphin promoter with a decrease of histone 3 lysine 27 trymethylation, associated with gene repression, in acute and dependent groups.
This data indicate a linkage between gene expression alterations and epigenetic modulation in prodynorphin promoter, thus suggesting a chromatin remodelling during ethanol exposure, as already proposed (Pandey et al. 2008).

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The phytoestrogen ferutinin counteracts the bone loss induced by ovariectomy in rats

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Osteoporosis is a chronic disease of the skeleton characterized by bone loss due to an imbalance between the two processes of bone formation and bone resorption. The most common cause of age-associated osteoporosis during menopause is the ovarian hormone deficiency. In this context the hormone replacement therapy (HRT) is the most common and effective strategy to prevent bone loss and skeletal fractures in post menopausal women, even if it is associated to negative side effect including increased risk of endometrial, ovarian, and breast cancer, thromboembolic events, and vaginal bleeding. For this reason, some natural substances of plant origin, showing the ability to prevent bone loss in both experimental and clinical studies, could represent the new strategies for the treatment of osteoporosis. Recently we reported that ferutinin, the main component of *Ferula hermonis* Boiss root, is able to prevent estrogen deficiency-induced bone loss in ovariectomized rats (Palumbo et al. 2009). Experiments performed *in vitro* showed that ferutinin is a potent phytoestrogen with high affinity for both subtypes of estrogen receptors (ERs), particularly for ERα (Ikeda et al. 2002). The aim of the present study was to evaluate the ability of ferutinin to counteract severe osteoporosis in ovariectomized rats, when daily administered by oral gavage for 30 and 60 days starting from 2 months after ovariectomy. Female Sprague-Dawley rats, aged 7 weeks, were divided into 4 groups of 10 animals each: 1) sham-operated (SHAM); 2) ovariectomized and treated with vehicle (C-OVX); 3) ovariectomized and treated with ferutinin at the dose of 2 mg/kg/die (F-OVX); 4) ovariectomized and subcutaneously injected with estradiol benzoate at the dose of 1.5 µg/rat/twice a week (EB-OVX). Half the animals of each group were sacrificed after 30 days of treatment, whereas the remaining ones were treated for 60 days. Soon after the sacrifice, the 4th and 5th lumbar vertebrae and the right femur were taken from each animal and processed following the common procedures for light microscopy observations. The 4th lumbar vertebrae were cut according to sagittal planes, whereas the 5th lumbar vertebrae were transversely cut; the distal epiphysis and the shaft of the femurs were sagitally and transversely sectioned, respectively. Histomorphometrical analysis of trabecular and cortical bone from lumbar vertebrae and femur respectively, demonstrated that ferutinin, like estradiol, induced a significant increase in BV/TV (trabecular bone volume) and Ct-B-Ar (cortical bone area) already after 30 days of treatment in comparison with C-OVX animals. Such effect of both ferutinin and estradiol benzoate on bone mass was more evident after 60 days of treatment.
These results clearly demonstrate the ability of ferutinin to recover the bone mass in osteoporotic ovariectomized rats, mimicking the effect of estradiol. The assessment of uterus and body weight as well as the histological analysis of the uteri were also included in the study.

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Pistacia lentiscus essential oil: molecular mechanisms of its antiproliferative activity

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The phytocomplex from *Pistacia lentiscus*, a shrub of the Anacardiaceae family, is an essential oil obtained by hydrodistillation of leaves, fruits or from a trunk exudate (mastic gum). The mastic gum has been known to be effective in several gastric diseases, against *Helicobacter Pylori* (Huwez et al. 1998) and for its antibacterial and antifungine activities. Furthermore, *Pistacia* oil's major chemical constituents are monoterpenes with chemiopreventive and chemiotherapic properties (Crowell et al. 1999).
We investigated the antiproliferative properties of the volatile oil from *Pistacia lentiscus* twigs and leaves using human cell lines from ovarian (2008 and C13*) and colon (LoVo) adenocarcinoma, and human stable fibroblast line (HFFF2) as *in vitro* models.
The MTT test (Mossmann 1983) showed that, after 3 h treatment, phytocomplex (about 150 µg/ml) was able to inhibit the growth of all adenocarcinoma cell lines. After 24 h treatment the IC_{50} on 2008 and LoVo cells resulted 3 times lowers. On fibroblast line the phytocomplex was active only after 72 h treatment (Casarin 2009). Western blot analysis (Zhang et al. 2003) confirmed the oil capability to reduce carcinoma cell growth by decreasing the expression of p-ERK, MAPKs induced by mitogenic stimuli.
Using Annexin V with propidium iodide we observed that oil was able to stimulate apoptosis in a dose-dependent manner. Analysis of mitochondrial membrane potential, with rhodamine123 (Xia et al. 2007), and reactive oxygen species (ROS) generation, with H_2DCF-DA (Lin et al., 2008), showed the oil capability to activate mitochondrial apoptotic pathway. Oil-treatment also induced alteration on H^+ gradient and interruption of electron flow between respiratory chain complexes III and IV, thereby causing loss of ATP (Lenaz 2005).
Our data also indicate that *Pistacia lentiscus* oil caused programmed cell death via a caspase-dependent pathway. In fact, after 3 h treatment and 21 h incubation, caspase 3 activity level, an apoptotic effector, resulted higher than the control one, especially for the highest dose used.
We also performed a flow cytometry-based cell cycle analysis, observing that the phytocomplex induced dose-dependent arrest in G_2/M phase by decreasing cyclin B1 levels (Hochegger et al. 2008) on all adenocarcinoma lines, especially on ovarian cells and acting on acetylated tubulin and microtubules' polymerization/depolymerisation.

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Quantitative analysis of regional left ventricular function in post-infarct mouse by magnetic resonance with retrospective gating and effect of pharmacological treatment with valsartan

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Murine models of myocardial infarction have been extensively studied as means of clarifying the functional, structural, and molecular changes associated with ischemic heart disease, in addition to testing the effectiveness of pharmacological therapies. Magnetic resonance (MR) imaging is the method of choice for the left ventricular (LV) volumetric quantification in clinical practice. The aim of this study was to test MR imaging in the quantification of global and regional LV function in mice with myocardial infarction, by using cine MR images.
Myocardial infarction was induced in 18 female C57BL6 mice by permanent ligation of the left anterior descending (LAD) coronary artery, as previously described (Tarnavski et al. 2004). Ten sham-operated animals were likewise treated, except that the ligature around the coronary artery was not tied. Seven days after surgery the cine short-axis images were obtained using a 4.7T vertical-bore MR magnet (Bruker) with retrospective gating (Intragate). The MR Dicom images were analyzed using custom software implemented in the Matlab environment. In order to obtain global indices, the left ventricular end-diastolic (EDV) and end-sistolic (ESV) volume were obtained as sum of areas in each slice multiplied by the slice thickness. In this way, we can calculate both stroke volume (SV) and ejection fraction (EF%). EDV and ESV were higher in ischemic animals than in sham-operated, whereas EF% was lower (EDV: 92±25 vs 36±4; ESV: 66±27 vs 10 ±3µl, $P<0.05$, EF% 31±10 vs 72±7%, respectively). To obtain an index of LV regional function we measured the regional fractional area change (RFAC%) as an index of regional mobility; this parameter is visualized with a bull's eye. We validated the method with histological analysis.
The renin-angiotensin system - particularly angiotensin II (AngII) - is involved in LV remodelling after myocardial infarction. Angiotensin-converting enzyme (ACE) inhibitors and AngII type 1 (AT_1) receptors blockers have been shown to decrease the mortality and morbidity of patients with myocardial infarction and heart failure (Pfeffer et al. 1992). Experimental evidence indicates that these drugs are effective in limiting post-myocardial infarction LV remodelling and failure (Schieffer et al. 1994). To evaluate the effect of valsartan, an AT_1 receptor antagonist, myocardial infarction was induced in 30 female mice. Twenty-four h later the heart was visualized with MR imaging and the functional parameters were calculated. Animals with EF% between 35% and 50% were included in the study and divided into two groups (7 animals each) and then treated with valsartan 1mg/kg/day or vehicle in drinking water. One, four, and eight weeks after surgery animals underwent MR imaging interrogation and functional parameters were calculated. After the last MR imaging acquisition the mice were anesthetized and the hearts were removed and processed for histological analysis.
The results indicate a beneficial effect of valsartan on EF%, infarct size, and collagen content.
ANOVA analysis showed a significant difference ($P<0.05$) in EF% evolution between controls and the treated group. On mid papillary slices, the infarct size (measured with ematoxilin/eosin staining) and collagen content (measured with Masson's trichrome staining), were higher in the vehicle group than in the valsartan group (infarct size: 52.4±10.7 vs 36.6±3.9%, $P<0.05$; collagen content 65.3±15.4 vs 46.3±73.9%, $P<0.05$).
On MR images at the end diastolic frame of the mid papillary slice, we measured the anterior and posterior wall thickness and ventricle diameter, which were smaller in the vehicle group than in the valsartan group (0.274±0.084 vs 0.429±0.189 and 0.208±0.166 vs 0.456±0.271 mm, $P<0.05$ for anterior and posterior wall thickness, respectively) and left ventricle diameter which was higher in the vehicle group than in the valsartan group (5.490±0.631 vs 4.940±0.635 mm; $P<0.05$). In conclusion, treatment with valsartan had a beneficial effect on LV functionality and on remodelling. Indeed, drug treatment, effectively inhibited LV dilatation, and reduced infarct size suggesting an important role of AngII in post-infarction LV remodelling.

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Expression and function of the $P2X_7$ purinergic receptor in monocytes from patients with Behçet's disease

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My doctoral work has dealt with the putative role of the $P2X_7$ purinergic receptor in modulating the inflammatory response in patients with Behçet's disease (BD).
The $P2X_7$ receptor, a member of the P2X family of nucleotide-gated channels, is a 595 amino acid polypeptide with two membrane-spanning domains and intracellular N- and C-terminal domains (Ferrari et al. 2006). In contrast to other members of the family, $P2X_7$ is predominantly expressed by mononuclear cells such as monocytes, macrophages, and dendritic cells (Ferrari et al. 2006; Gu et al. 2000) and seems particularly involved in the inflammatory response triggered by the cell damage throughout the release of ATP (Di Virgilio et al. 2007). In fact, the $P2X_7$ receptor plays a key role in promoting the release of pro-inflammatory cytokines, including IL-1β, IL-18, and IL-6 (Di Virgilio et al. 2007; Caporali et al. 2008).
BD is a systemic autoimmune inflammatory disorder characterized by vasculitis that is often accompanied by oral and genital ulcers, skin lesions, uveitis, and arthritis (Yurdakul et al. 2004). Its typical course consists of sudden recurrent inflammatory attacks intercalated with periods of remission. Notably, the pro-inflammatory cytokines modulated in an excitatory manner by the $P2X_7$ receptor seem to play a relevant role also in BD, as demonstrated by the presence in such patients of either an elevated IL-1β concentration in the synovial fluid (similar to that of rheumatoid joints) (Pay et al. 2006), and a significantly higher level of IL-18 in the serum and the bronchoalveolar lavage with respect to healthy controls (Oztas et al. 2005; Hamzaoui et al. 2003). On this basis, we hypothesized that $P2X_7$ receptor could be involved in the patogenesis of BD, and then we decided to study its expression and function in the peripheral monocytes from BD patients.
The monocyte surface expression (percentage of positive cells and mean fluorescence intensity, MFI) of $P2X_7$ was determined by flow cytometry from BD patients and healthy controls. Despite the high variability in the expression level of $P2X_7$ among the whole study population, we observed that MFI was significantly higher in cells from BD patients compared with controls.
The function of $P2X_7$ was assessed by analyzing the effect of the $P2X_7$ receptor agonist 2'-3'-O-(4-benzoylbenzoyl)ATP (BzATP) on Ca^{2+} fluxes in monocytes using a single-cell fluorescent microscopy method. The effect of BzATP in stimulating Ca^{2+} influx is significantly higher in monocytes from BD patients than healthy controls.
In conclusion, these preliminary results suggest a possible involvement of $P2X_7$ in the pathogenesis of immuno-inflammatory activation observed in BD. On this basis, is our intention to confirm the above results: i) using qRT-PCR and Western blotting to further assess $P2X_7$ expression; ii) evaluating $P2X_7$ function also by studying cytokine production, pore formation, and induction of apoptosis in monocytes stimulated with BzATP.

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Effects of genistein in Leber's hereditary optic neuropathy (LHON) cell models

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Leber's hereditary optic neuropathy (LHON), a maternally inherited form of central vision loss, is associated with mitochondrial DNA pathogenic point mutations affecting different subunits of complex I, the first site of the mitochondrial respiratory chain. G11778A, T14484C, and G3460A are the most frequent and studied Leber's mtDNA mutations. LHON is considered a pathology at variable penetrance: the mutation of mtDNA, even if necessary, is not sufficient to induce the evident clinical symptoms. Proposed genetic modifiers include heteroplasmy, secondary mtDNA mutations, haplogroup, nuclear encoded genes, tobacco and alcohol consumption, and the exposure to toxic compounds. Unfortunately at present there are not treatments for LHON.

Approximately 50% of males and only 10% of females harbouring a primary mtDNA mutation develops optic neuropathy and we observed that estrogens might positively prevent cell damage, so here we investigate the effect of phytoestrogens, i.e. genistein, one of the soy isoflavones.

LHON cybrids are cytoplasmic hybrid cells obtained by fusion of LHON patient enucleated fibroblasts and mtDNA-depleted osteosarcoma cells (rho0 cells), thus excluding the influence of LHON donor nuclear genome. To study genistein protection from oxidative stress, cybrids were incubated in galactose medium (glucose free), forcing the cells to rely mainly on the mitochondrial respiratory chain to produce ATP. Results show that, contrary to control cybrids, in each cell line harbouring one of the three most frequent LHON pathogenic mutations, ROS increase, mitochondrial network is altered and there is a high percentage of cell death when galactose replaces glucose in the medium. Genistein (0.1 μM) prevents the alterations induced by galactose on cell growth, ROS production, and mitochondrial network. Furthermore genistein activates ERK1 and ERK2 MAP kinases thus up-regulating Mn-SOD antioxidant enzyme. This higher expression of antioxidant proteins leads to a decrease in ROS levels and subsequent cell protection. The effects of genistein are antagonised by ERs specific inhibitor (ICI 182780), demonstrating the involvement of estrogens' receptors as mechanism of protection of LHON cybrids from oxidative stress.

Neuronal environment influences endothelial angiogenic functions and vessel integrity

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In the central nervous system (CNS), maintenance of vascular integrity is essential to brain homeostasis under physiological conditions (Murakami et al. 2008). The cross-talk between neurons and vascular endothelial cells is strictly controlled, and both components participate to blood brain barrier (BBB) organization (Abbot et al. 2006).
Defects in vascular integrity are associated with several human pathologies of CNS, such as vascular malformation, hemorrhagic stroke, and Alzheimer's disease (AD; Dejana et al. 2009). Frequently coexisting with AD, cerebral amyloid angiopathy (CAA) is characterized by deposition of amyloid peptides around brain vessels, recapitulating the main features of cerebrovascular pathologies, as barrier deficit and aberrant vessels. CAA is primarily an age-related disease, occurring in the sixth decade of life and therefore appears to be a suitable model for exploring the relationship between aging and the development of cerebrovascular diseases (Donnini et al. 2010).
The aim of this study was to investigate the role of neuronal components on vascular functions.
First, we showed that amyloid peptides (Aβ) administration impaired angiogenesis by causing endothelial cells to enter senescence at an early stage of vascular development in the zebrafish model. Then, in order to substantiate the premature senescence induced by Aβ treatment, we assessed β-galactosidase (β-Gal) activity on larval zebrafish (7 days post-fertilization). This analysis showed that Aβ peptides increased β-Gal and particularly at the level of pronephric ducts and axial vessels. At the end, we evaluated the activation of p21, a premature senescence-associated signal, on treated embryos at 72 h post-fertilization through *in situ* hybridization. Data confirmed that Aβ treatment increased p21 transcription, preventing the correct development of vessels.
We also performed *in vitro* co-culture experiments in order to investigate the influence of neuronal environment on the maintenance of vascular integrity. The angiogenic properties of endothelial cells was evaluated in a co-culture model, using transwell technique with human umbilical vein endothelial cells (HUVEC) and neurons, isolated from the cortex of mice embryos. Neuronal differentiation was completed at 10 days of culture as assessed by immunofluorescence analysis of specific markers (β3-tubulin, synapsin, and NMDA-R). HUVEC and cortex neurons (CNs) were co-cultured for 2-7 days, evaluating the formation of neuronal network (β 3-tubulin labelling), while we observed formation of HUVEC sprouts on gelatin-beads. Immunofluorescence assay on CNs showed that endothelial cells were able to support and accelerate neuronal differentiation and maturation, while CNs inhibited endothelial sprout formation. Then, by using neuronal conditional medium (CM) at 10 day of CNs culture, we evaluated endothelial angiogenic properties, such as the capability of HUVEC to grow, migrate and form sprouts and branching. We observed a reduction of HUVEC proliferation (measured by BrdU uptake) and a reduction of capability to migrate and to form pseudocapillaries. Moreover, CM was devoid of apoptotic activity, promoting cell survival through increased phosphorylation of Akt and caspase-3 inactivation. We then focused on the expression of TJ proteins (occludin and claudin-3), in order to assess BBB vascular integrity in response to neuronal CM. Immunofluorescence and Western blot analysis of TJ proteins revealed an increased expression of these proteins in HUVEC, which was confirmed by functional test of permeability assay, documenting a decrease of CM-treated HUVEC permeability.
In the complex our results suggest that neurons and endothelium interact, modifying some characteristics like neuronal maturation, angiogenic properties and vascular integrity. In particular, neuronal environment has an inductive influence on endothelial cells, promoting the acquisition of the expression of some characteristics typical of BBB endothelium.
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Role of "diazepam binding inhibitor" in oxygen-glucose deprivation and reperfusion-induced damage in rat brain slices

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During brain ischemia, tissues subjected to oxygen/glucose deprivation (OGD) undergo within seconds to disruption of the energy supply, which leads to massive cell depolarisation. These results in a quick rise of extracellular glutamate caused by reversed operation of neuronal glutamate transporters, formation of free radicals, mitochondrial damage, and fragmentation of cellular DNA (Saito et al. 2005). GABA and GABA agonist compounds as well, represent physio-pharmacological means to inhibit cellular activity within the brain, possibly blunting ischemic injury (Ricci et al. 2007; 2009). Furthermore protein expression changes so greatly at the early time point of reperfusion that it provides important molecular information about the initiation of ischemia–reperfusion injury cascade (Prajapati et al. 2010).
A proteomic profiling using two-dimensional gel electrophoresis was performed by comparing samples of rat brain slices subjected to control conditions or OGD and reperfusion (OGD/R). Results indicated that 16 spots out of the1550 were differently expressed and among these, the diazepam binding inhibitor (DBI) was found to be completely absent in the OGD samples. Consequently, the role exerted by DBI (0.001-10 μM) in OGD/R related damage was investigated in rat brain slices. Its neuroprotective/damaging effect was assessed by measuring the release of glutamate and lactate dehydrogenase (LDH) during reperfusion and by determining final tissue water gain, taken as an index of cell swelling.
The results showed that, in control conditions, DBI caused a significant increase in glutamate release at the highest concentration used while it did not modify LDH and tissue edema. On the contrary, when the peptide was added to artificial cerebrospinal fluid used during reperfusion, a significant LDH release was observed at DBI concentrations of 0.001-0.1 μM, while higher concentrations were ineffective. Moreover DBI significantly increased glutamate release as well as tissue water gain although according to a "bell-shaped", hormetic-like, concentration-response curve, with an efficacy window of 0.1-1 μM.
The receptors involved in DBI-mediated effects were further investigated by using selective antagonists for central and mitochondrial DBI receptors (flumazenil and PK-11195, respectively). Flumazenil antagonised in a concentration-dependent fashion DBI-induced LDH and glutamate release and tissue edema while PK-11195 had no effects, suggesting that benzodiazepine central receptors are involved in DBI effects.
In conclusion, these results open new fields of study designed to better characterize DBI mechanism of action with the objective to delineate a therapeutic strategy useful to prevent and/or reduce ischemia and reperfusion-induced tissue damage.
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Myocardial functional remodelling in familial hypertrophic cardiomyopathy: impact on arrhythmogenesis and contractile function

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Hypertrophic cardiomyopathy (HCM) is associated with a severe risk of ventricular arrhythmias and sudden death. However, the exact mechanisms leading to the increased arrhythmogenic of HCM myocardium are still unknown and no specific drug aimed to selectively prevent arrhythmias in HCM patients exists.
Myocytes were enzymatically dissociated and trabeculae dissected from samples of septal tissue harvested from HCM patients undergoing myectomy.
In HCM human myocardium a prolongation of action potential duration (APD) was found. Decreased repolarising currents density (reduced I_{to}) and altered potassium channels expressions seem to be responsible for the prolonged action potential. Prolonged action potential, by increasing the risk of early after depolarization, may provide a substrate for arrhythmias in diseased myocardium.
We then investigated how changes in action potential dynamics affect sarcolemmal Ca^{2+} fluxes and contractile performance of HCM tissue.
In HCM vs control septal myocytes I_{CaL} amplitude and kinetics were unchanged while Na^+/Ca^{2+} exchanger (NCX) expression was increased ~2 fold. In HCM cells, an I_{Na}-late blocker, ranolazine, reduced APD by ~30%, while it had no effects on control cells, suggesting an up regulation of I_{Na}-late current in HCM tissue. Increased I_{Na}-late current not only prolongs action potential but also slows down calcium extrusion via NCX, contributing to Ca^{2+} overload. Ranolazine applied on HCM cells reduced Ca^{2+} transient amplitude and shortened its duration, likely improving Ca^{2+} extrusion through NCX. Ranolazine also contributed to reduce diastolic calcium levels, proving effective in ameliorating intracellular calcium overload in diseased cells. Ranolazine applied to HCM intact ventricular trabeculae: i) reduced active twitch tension and diastolic tension at high stimulation rates; ii) tended to speed up relaxation.
In HCM myocardium, NCX over expression and Na^+ accumulation due to increased I_{Na}-late may promote a large increase of Ca^{2+} entry trough NCX reverse mode, which contributes to prolong APD and may increase Ca^{2+} transients' amplitude and duration. Ranolazine, by reducing intracellular Na^+ overload, potentiates Ca^{2+} extrusion through NCX and thus has a negative inotropic effect and may ameliorate diastolic function in patients. Moreover, by shortening AP duration, ranolazine might represent a selective drug to reduce the arrhythmogenic burden in HCM patients.

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Adenosine is released *per se* in physiological conditions. During ischemia it is primarily a product of extracellular ATP

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There has been increased interest in the last years of the past century on the role of purines, adenosine, and adenosine triphosphate (ATP), that are important intracellular inter-convertible metabolites. The role of ATP as a neurotransmitter has been largely demonstrated both in the peripheral and the central nervous system (CNS) (Burnstock 2007). In the brain, ATP is released from neurons (Khakh et al. 1998) and astrocytes (Koizumi et al. 2003) through various mechanisms, a vesicular mode (Sperlagh et al. 1998) or along an electrochemical gradient through connexin hemichannels (Goodenburg et al. 2003) or maxi-anionic membrane channels (Bodin et al. 2001) or even through P2X7 channel (Suadicani et al. 2006). Adenosine is considered one of the most important modulators of neurotransmission (Sebastiao et al. 2000; Latini et al. 2001). Despite a vast literature on the modalities of release of adenosine and ATP from CNS cells (Latini et al. 2001; Burnstok 2007), it is still unknown which part of adenosine derives extracellularly from released ATP. Enzymes ectonucleotidases are present extracellularly, membrane bound and may account for ATP conversion to adenosine (Zimmerman 2000).
The objective of this study was to investigate the contribution of extracellular ATP to the adenosine formation under basal (i.e. physiological) and ischemic conditions *in vivo* by using a new inhibitor of ecto-ATPases, $[TiW_{11}CoO_{40}]^{8-}$ (PV4), which is the most potent inhibitor of different ecto-ATPase inhibitors, polyoxometalate compounds, recently synthesized by Muller and coworkers (2006). Extracellular adenosine and ATP were measured in striatal microdialysates by HPLC and by luciferine/luciferase, respectively. Such assays let to detect concentrations present in basal samples (fentomoles).
Under physiological conditions (i.e. when neurons spontaneously fire), in the presence of PV4, extracellular concentrations of adenosine (mean value of 3 h 140.0±2.0 nM, n=5) did not decreased indicating that adenosine is released *per se* from cells. Extracellular concentrations of ATP in the presence of PV4 increased by 12 fold (mean values from 30.0±4.0 to 360±53 nM, n=4-6). Therefore, although more than 90% ATP is hydrolyzed, it remains in the intermediate products ADP and AMP without further conversion to adenosine.
Under focal ischemia induced by medial cerebral artery occlusion, and in the presence of PV4, extracellular ATP concentrations increase from 50 nM to 450 nM while adenosine decreases from 690 nM (n=5) to 275 nM (n=10). Therefore 60% of extracellular adenosine is accounted by ATP hydrolysis and 40% is released *per se*. A time-related analysis of the origin of extracellular adenosine indicates that a prompt degradation of ATP followed by adenosine formation occurs in the first 20 min after ischemia.
Degradation of ATP by ecto-nucleotidases after ischemia represents an important mechanism in the regulation of ATP and extracellular levels of adenosine in the first hours after ischemia. In view of the important roles of both adenosine and ATP in ischemia, such information is important in devising putative purinergic strategies aimed at protection from ischemic damage.

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GARDA study: adverse drug reactions in geriatric wards of Verona hospital

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Adverse drug reaction (ADR) are considered to be among the leading causes of morbidity and mortality, in particular older patients are vulnerable to ADRs because of multiple-drug regimen and aged-associated changes in pharmacokinetics and pharmacodynamics. In elderly patients 16.6% of hospital admissions are due to ADRs (Berijer et al. 2002), and 19-23% of hospitalized experience ADRs, causing significant prolongation of hospital stay (Leach et al. 1986; Bowman et al. 1996). Moreover, fatal ADRs are estimated to occur in 0.31% of hospitalized patients, and complications from drug therapy constitute the most common adverse events in hospitalized patients (Leape et al. 1991). The aims of this study was to evaluate in an elderly population: i) the incidence of ADR-related hospital admission; ii) the drugs involved; iii) the incidence of ADR during hospitalization; iv) the drugs which have been identified as having causal relationship with ADRs. Another aim was to control the effectiveness of spontaneous reporting system in Geriatric Units. The study, lasting six months, was conducted in three geriatric wards of Verona hospital. For each patient, at the admission and during the hospitalization, physicians and nurses registered the ADRs and drugs responsible. Health staff was also requested to report ADRs to the Italian Pharmacovigilance System. In 11% of 1023 studied patients, the hospital admission was caused by an ADR. The patients which experienced an ADR during the hospitalization were 256 (25%). The percentage of admissions due to ADRs is higher in women (12.1%) than men (10.1%), although the difference was not statistically significant (RR 1.19; 95% CI 0.84-1.69). Also the incidence of ADRs during hospitalization is higher in women (27.1%) than in men (22.7%), close to statistical significance (RR 1.19; 95% CI 0.96-1.47). The ADRs that most frequently led to hospitalization were the electrolyte alterations: hyponatremia was the most reported, followed by hypokalemia and hyperkalemia. During hospitalization the electrolyte abnormalities were also the most frequent ADRs, albeit with a different order: hypokalemia, followed by hyponatraemia and hyperkalemia. The drugs most involved in the development of ADRs causing hospitalization were diuretics, followed by antiplatelet agents and ACE inhibitors.

During hospitalization the drugs most involved in ADRs were, once again, diuretics, followed by anticoagulants and antibacterials. During the six-month period of the study, the Pharmacovigilance System received 32 reports of ADRs coming from the geriatric wards (underreporting=91.3%).

In conclusion a significant incidence of ADRs leading to hospital admission or occurring during hospitalization was found among elderly people. Many of the implicated drugs in ADRs were old drugs. Our study showed that most of these ADRs are potentially preventable and that the vigilance by clinicians and nurses in detecting, diagnosing, and reporting ADRs is important for continued drug safety monitoring.

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Reduction of SNAP25 expression enhances neuronal calcium currents and network activity

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SNAP25 is a member of the SNARE protein complex that participates in synaptic vesicle exocytosis. We previously demonstrated that differential SNAP25 expression in hippocampal neurons regulates intracellular calcium dynamics (Verderio et al. 2004) and that negative modulation of neuronal voltage-gated calcium channels requires SNAP25 activity-dependent phosphorylation on Ser187 (Pozzi et al. 2008). Since modifications of the protein expression levels have been reported to occur in different brain diseases, including schizophrenia, ADHD, and epilepsy, we used SNAP25$^{+/-}$ mice to investigate whether reduction of SNAP25 expression induces altered network activity *in vitro* and *in vivo*, possibly leading to neurological phenotypes. A significant increase in calcium responsiveness to depolarization was observed in SNAP25$^{+/-}$ neurons as well as in synaptosomes from heterozygous mice relative to wild-type. Also, increased voltage-gated calcium currents were recorded in excitatory but not inhibitory neurons. Behavioural tests on wild-type and SNAP25-heterozygous mice revealed an increased spontaneous motor activity and a deficit in long term memory in SNAP25$^{+/-}$ mice; furthermore, *in vivo* electroencephalographic recordings from both cortex and hippocampus showed a significant increase in the number of spikes in SNAP25$^{+/-}$ mice. Treatment of SNAP25$^{+/-}$ mice with antiepileptic drugs, such as ethosuximide and valproic acid, caused a significant reduction in the number of cortical spikes recorded by EEG. These data suggest that a dysregulation of calcium dynamics, due to alterations of SNAP25 expression, may lead to unbalances of neuronal network activity, possibly associated to several neurological diseases.

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Molecular and functional studies of receptor enhancing expression protein (REEP1) involved in hereditary spastic paraplegias

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Hereditary spastic paraplegias (HSPs) are a clinically and genetically heterogeneous group of conditions that are characterized by the presence of lower limb spasticity and weakness. The common pathological feature of these conditions is retrograde degeneration of the longest nerve fibres in the corticospinal tracts and posterior columns (Salinas et al. 2008).
HSP can be inherited as an autosomal dominant, recessive, or X-linked recessive trait, and at least 41 spastic paraplegia gene (*SPG*) loci have been mapped and 33 genes identified to date. Autosomal dominant HSP is the most prevalent form and represents around 70% of cases (Zhao X et al. 2009). Several mechanisms have been postulated to explain motor neuron death in HSP, however, none of these is completely satisfactory to elucidate the entire process or support a specific therapeutic strategy. The objective of this work is to understand the molecular function of the newly identified receptor enhancing expression protein (REEP1), the third cause of ADHSP (autosomal dominant hereditary spastic paraplegia; Hewamadduma et al. 2009) using different cell cultures: Hela and Cos7 cells to understand the biological mechanism of REEP1, and neuronal stem cell (NSC) as a model system to analyze neuronal phenotypes produced by dysfunction in REEP1.
REEP1 gene consists of seven exons and codifies for a protein of 202 amino acids with a molecular weight of 22.25 kDa. REEP1 contains two predicted transmembrane domains (TM1 and TM2) and a domain of unknown function called "deleted in polyposis" (TB2/DP1/HVA22), a protein which in humans is deleted in severe forms of familial adenomatous polyposis, an autosomal dominant oncological inherited disease (Beetz et al. 2008).
In order to search the subcellular expression profile of REEP1 and its topology we generated different constructs with the HA and Myc tags fused to the N-terminal and C-terminal part of REEP1. Immunofluorescence experiments and confocal imaging analysis of Cos7 cells expressing these constructs showed that REEP1 is an integral membrane protein that localized to the endoplasmic reticulum (ER) membrane. We also analyzed the orientation of REEP1 in the lipid bilayer of ER by a selective permeabilization of cellular membranes using digitonin and found that the C-terminal domain of REEP1 is orientated to the cytoplasm.
When the protein is overexpressed in cells, we also observed that REEP1 presents aggregates that co-localize with markers of both ER and Golgi membranes. Results indicate that, in this condition, Golgi is fragmented, suggesting profound alterations in the control of intracellular proteins traffic.
Moreover, to study the pathological mechanism of HSP, we have reproduced in the wild type REEP1 protein two pathological mutations found in patients: A20E and P19R. Future experiment will be focused on understanding the cause of the aggregates produced by REEP1 overexpression and on analyzing the consequences of pathological mutations of REEP1 as well as on generating a neuronal cell model using NSC cells in which reproduce the mutations previously tested in Hela and Cos7 cells.

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Neuropeptides effects on aminergic release in the hypothalamus

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The regulation of food intake and energy expenditures is integrated in the hypothalamus, where peripheral hormone and neurotransmitter signalling convey timely updated information about energy needs and metabolic substrate availability (Karla et al. 1999). Our experiments aimed to investigate the possible role of several neuropeptides, such as agouti related protein (AgRP, anorexigenic), melaton-II (MT-II, orexigenic), endomorphins (Ems, anorexigenic), on catecholamines and serotonin release in the hypothalamus. Noradrenergic fibers arising from *nucleus tractus solitarius* innervate the hypothalamus, where α_1- and α_2-adrenoceptors mediate anorectic and feeding stimulatory behaviour, respectively (Wellman et al. 1993). We have previously observed that anorectic peptides such as leptin inhibit depolarization-induced norepinephrine release in the hypothalamus (Brunetti et al. 1999). The role of dopamine in the control of feeding in the hypothalamus is still unsettled, with studies showing both inhibitory and stimulatory effects. On one side, the classical anorectic agents, such as amphetamine, are supposed to produce their effects through dopamine reuptake inhibition (Samanin et al. 1993); on the other hand, dopamine administration into the lateral hypothalamus increases food intake (Shiraishi 1991) and raised dopamine levels are found in obese animals (Yang et al. 1995). This could be explained by the opposite effects of dopamine D_1 and D_2 receptors on orexin neurons in the lateral and perifornical hypothalamus. It is well known that serotonergic fibers arising from the dorsal raphe nucleus in the brain stem and projecting to the hypothalamus play a key role in regulating feeding behaviour, as evidenced by the anorectic effect of fenfluramine and dexfenfluramine, both in rodents and in humans. In particular, direct microinjection of serotonergic agents in hypothalamic paraventricular and ventromedial nuclei inhibit food intake (Leibowitz et al. 1990). We have previously reported that the feeding stimulatory role of orexin peptides could be related to their inhibition of hypothalamic serotonin release (Brunetti et al. 2001). In order to further elucidate the role of neuropeptides in the feeding regulatory pathways, we have evaluated their effects on neurotransmitters release from rat hypothalamic neuronal endings (synaptosomes) *in vitro*, both basally and during a mild depolarization stimulus (15 mM K^+). Synaptosomes were prepared according to Gray et al. (1962), layered onto 0.8 μM Millipore filters and perfused into 37°C water-jacketed superfusion chambers. AgRP and MT-II are melanocortin peptides with opposite effects on feeding regulation; our findings, which show that they do not modify both basal and depolarization-induced neurotransmitter release from hypothalamic synaptosomes, rule out catecholamines and serotonin as mediators of AgRP and MT-II effects. The importance of opioid system in feeding behaviour is well documented: opioid receptor agonists stimulated food intake, while opioid receptor antagonists, especially the μ- and κ-antagonists, induced decreases in feeding and body weight, in rodents. Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2; EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2; EM-2) combine potency and efficacy with high affinity and selectivity towards μ receptor. The EMs have three critical elements: N-terminal sequence, peptide length, and C-terminal amidation. Endomorphin-1, in the dose range 0.1-100 nM, did not affect basal dopamine and serotonin release. On the other hand, endomorphin-1 significantly stimulated basal norepinephrine release. In a second set of experiments, we observed that this peptide did not modify the stimulated release of dopamine, norepinephrine and serotonin. Endomorphin-2, in the dose range 0.1-100 nM, did not affect both basal and depolarization-induced release of catecholamines and serotonin. Our findings showing that endomorphin-1 increased basal norepinephrine release from hypothalamic synaptosomes, leaving unaffected either basal or depolarization-induced dopamine and serotonin release, support a role for norepinephrine in mediating the orexigenic effects of endomorphin-1.

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Role of GILZ and L-GILZ, a new isoform, in the regulation of T Cell growth

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Glucocorticoids (GCs) play a role in the physiologic regulation of immune system and are commonly used as therapeutic agents in the pharmacological suppression of inflammatory response, through molecular mechanisms involving glucocorticoid receptor activation and modulation of gene expression. The immunosuppressive activities of GCs mainly affect T lymphocytes. In fact, GCs modulate T lymphocyte activation, proliferation, apoptosis, contribute to thymic selection, and also regulate cytokine production, thus modulating T helper cells differentiation.
GCs modulate transcription of numerous genes including glucocorticoid induced leucine zipper (GILZ). GILZ, discovered in our laboratory, is a rapidly dexamethasone (DEX) induced gene that was shown to mediate several GC functions, such as modulation of T lymphocytes activation, IL-2 production, apoptosis, and cell proliferation. Many molecular targets of GILZ have been identified, including NF-κb, AP-1, Raf-1 and Ras, all involved in GC effects. Recently we have cloned a new GILZ isoform of 705 bp, coding for a protein of 234 amino acids, named long-GILZ (L-GILZ).
Structural analysis showed that these proteins have in common several domains: TSC box, leucine zipper, and PER region, while they differ in the N-terminal domain.
We performed experiments to analyze the GILZ/L-GILZ system role in the control of immune response.

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Electroencephalographic and behavioural alterations in a model of focal cortical dysplasia in rats

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Epilepsy is a chronic neurological condition, characterized by recurrent and spontaneous seizures that afflict over 50 million people worldwide: 30% of afflicted shows drug-resistance and about 60% of drug-resistant epilepsies are associated with cortical focal lesions, due to defect of cortical maturation (dysplasia) (Mischel et al. 1995).
The epileptogenic mechanisms underlying these malformations are still poorly understood and the available animal models are not yet fully characterized (Colacitti et al. 1999).
The purpose of the present study was therefore to investigate, from the behavioural and electroencephalographic point of view, a model of focal cortical dysplasia (FCD), which mimics the human pathology. This model is obtained with rats whose mother was previously treated with carmustine (BCNU) at embryonic-day 15 (Bernadete e Kriegstein 2002). The animals were tested at two different ages (3 and 6 months) to assess the kinetic of the alterations.
Firstly motor integrity, through the evaluation of spontaneous motor activity, expressed in terms of total number of vertical and horizontal movements, was investigated. Long-term aversive memory was evaluated through the passive avoidance test. Spatial short and long term memory was investigated using the 8-arm radial maze. EEG was evaluated in freely moving awake rats one week after the insertion of four electrodes in the parieto-occipital cortex, for 1 h, through a Powerlab system.
The results showed a significant decrease of horizontal movements only in BCNU-treated group 6 months old ($P<0.05$, two-way ANOVA). Both short and long term spatial memory, at both ages, were impaired in BCNU treated rats ($P<0.001$, two-way ANOVA) in terms of increased number of errors and change of strategies in the maze. No impairment in aversive memory was shown. EEG, recorded in BCNU-treated rats, showed some abnormalities in comparison with control group consisting of spike activity at the age of 3 months and more evident at the age of 6 months ($P<0.001$, two-way ANOVA), without showing an epileptic phenotype.
Our results show that pre-natal injection of carmustine in pregnant female rats may represent in offspring a valid animal model of human FCD characterized by cognitive deficit and EEG alterations even if this model must be convalidated by histopathological examination of the cortex and hippocampus.

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Regulation of COX-2 expression in colon cancer cells co-cultured with human platelets

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Cyclooxygenase(COX)-2, a key enzyme in the prostanoid biosynthesis, results to be up-regulated in human colorectal carcinomas and adenomas (Brown et al. 2005). In different cell types, it has been demonstrated the ability of activated platelets to promote COX-2 over-expression through different mechanisms, i.e. direct cell-cell interaction or soluble factors (Eligini et al. 2007; Dixon et al. 2006; Caughey et al. 2001; Goppelt-Struebe at al. 2000). Thus, the aims of this study were to investigate: i) the role of isolated human platelets on COX-2 induction (at transcriptional and post-transcriptional levels) in human colon cancer cell line (HT-29); ii) the possible role of platelet-derived prostanoids on COX-2 protein expression in HT-29 cells; iii) the consequence of platelet/HT-29 interaction on the release of prostanoids and angiogenic mediators. Co-culture experiments were performed with HT-29 cells ($1x10^6$) and human platelets($1x10^8$) isolated from whole blood of healthy volunteers, and it was assessed: i) COX-2 mRNA and protein expression, by real time-PCR and Western blot techniques, respectively; ii) prostanoids (thromboxane (TX)-B_2, prostaglandin (PG) E_2 and $PGF_{2\alpha}$), anti- and pro-angiogenic mediators (endostatin, VEGF, PDGF-BB) generation, by RIA and ELISA. Platelets caused a 2-fold increase of HT-29 COX-2 mRNA levels at 2, 4, and 20 h of co-culture, whereas there was a 3-fold increase of COX-2 protein only after 20 h. TXB_2 was the major prostanoid produced (86±19 ng) vs PGE_2 (2.5±0.6 ng) and $PGF_{2\alpha}$ (3.6±0.6 ng). Aspirin-treated platelets did not produce prostanoids but still caused a similar induction of COX-2 in HT-29 cells. COX-2 induction in HT-29 cells was found also using a cell transwell (pore size 0.4 µm) to avoid direct cell-cell interaction, and by platelet releasate. Incubation of HT-29 with platelets or platelet releasate caused an increase of COX-2 mRNA levels up to 24 h in the presence of transcriptional blockage by actinomycin D (1.25 µg/ml), suggesting a stabilization of the COX-2 transcript. In HT-29 cells co-cultured with platelets, the ratio between cytoplasmic and nuclear HuR (a COX-2 mRNA-stabilizing protein) assessed by confocal microscopy was significantly increased with respect to HT-29 cells cultured alone (0.40 pixel sum vs 0.26 pixel sum, $P<0.01$).
In summary, platelets induced COX-2 mRNA and protein expression in HT-29 cells. This effect did not involve either platelet-derived prostanoids or direct interaction between platelets and HT-29 cells. In contrast, a factor released from platelets may play a role through a post-transcriptional mechanism involving COX-2 mRNA stabilization by HuR translocation into the cytoplasm. In conclusion, these experiments enlighten the important role of platelets in tumorigenesis and will open the way to novel therapeutic strategies in chemoprevention.

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Pharmacogenetics research and its applications in current clinical practice

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The general aim of the PhD project is to provide evidence, in a translational perspective, of the application in the current medical general practice of the feasibility of pharmacogenetics to improve the appropriateness of drugs' prescription. The study involves two different approaches, the pharmacogenetics of the effects of antidepressant drugs in cancer patients and the pharmacogenetics-based prediction of warfarin dosage in oral anticoagulant therapy.
The research protocols of the two studies have been previously approved by ethical committee of the ASS1-Trieste.
Warfarin is a drug widely used for the oral anticoagulant therapy; however, warfarin therapy is difficult to manage due to the drug's narrow therapeutic index and the wide inter-individual variability observed in patients' response. Recent evidence has shown that single nucleotide polymorphisms (SNPs) in genes affecting warfarin metabolism (CYP2C9) and pharmacodynamic response (VKORC1) are strongly associated with warfarin responsiveness (The International Warfarin Pharmacogenetics Consortium 2009). In particular, two allelic variants, CYP2C9*2 and CYP2C9*3, have altered catalytic properties, which affect warfarin metabolism, and induce a reduced or a minimal enzyme activity, respectively (Taube et al. 2000). Within the SNPs identified in VKORC1, a single variation in position -1639 (G>A) can explain the variability in the dose required for an adequate oral anticoagulation (Limdi et al. 2010). The PhD project is aimed, therefore, to the analysis of the SNPs in CYP2C9 and VKORC1, as well as to the identification and validation of the algorithms of prediction of warfarin dose based on both pharmacogenetics and clinical factors. The patients have been recruited at the Centro Cardiovascolare and Distretto 1 (ASS1-Trieste). For each subject a salivary sample and the clinical records useful to integrate the genetic and clinical data to identify an algorithm of prediction of warfarin dose have been obtained. The activity performed so far has permitted to collect and genotyped the first group of 35 samples, and a database of clinical and genetics data has been created. At the moment, explorative statistic has been carried out but inference of the data has not been yet performed.
The genetic polymorphism of serotonin transporter 5-HTTLPR, which is the molecular target of SSRI antidepressants drugs, is endowed with a high penetrance and functional role, and significantly contribute to the therapeutic response and incidence of adverse effects (Serretti et al. 2007; Luddington et al. 2009). The aim of the PhD project is therefore that to examine the effects of SSRIs antidepressants drugs in relation to the 5-HTTLPR genetic polymorphism of serotonin transporter. The study is being performed in patients who are recruited by the palliative care team at different hospices. The patients have been treated with one of the three SSRI antidepressants drugs sertraline, citalopram or escitalopram. All the patients recruited have been psychologically evaluated at the admission and recruitment into the study and after 14 days of antidepressant treatment, using the Hospital Anxiety and Depression Scale (HADS) (Zigmond et al. 1983) and the Mini-Mental Adjustment to Cancer (Mini-MAC) (Watson et al. 1994). From each patient, a salivary sample has been collected in order to provide the genetic sample, which is been analyzed for the 5-HTTLPR by means of standard techniques. The statistical analysis have been carried out both considering the antidepressant effect of each SSRI drugs and the differences of the three drugs. The results obtained indicate that SSRIs are effective in attenuating depression and anxiety in the palliative care of advanced cancer patients, and that their effects are exerted also on the mental adaptation to cancer of these patients; these findings significantly depend of the genetic characteristics of the patients.

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Protective effect of CBD, a non-psychotropic cannabinoid, in DNBS-induced ulcerative colitis in mice

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Inflammatory bowel disease (IBD) refers to two chronic diseases that cause inflammation of the intestine: ulcerative colitis and Crohn's disease. IBD is relatively common in the U.S.A., accounting for disease in approximately 1 million individuals, with similar numbers in Europe (Lichtenstein et al. 2006). Although the incidence rates are beginning to stabilize in industrialized countries, rates continue to rise in low incidence areas such as Asia and most developing countries (Colombel et al. 2008). Pharmacological treatment, which includes aminosalicylates in ulcerative colitis, and corticosteroids, thiopurines, and anti-TNF therapies in both Crohn's disease and ulcerative colitis, is disappointingly unsatisfactory (Kozuch et al. 2008); therefore, new therapeutic approaches are required.
Cannabidiol (CBD), a plant-derived cannabinoid devoid of psychoactive properties, has been recently reported to exert pharmacological activities (antioxidant, anti-inflammatory, and immunomodulatory effects) and mechanisms (i.e. FAAH inhibition) of potential therapeutic interest for the treatment of gastrointestinal diseases (Izzo et al. 2009). Therefore, we investigated the effect of CBD in a chemical model of colitis. Colitis was induced in male mice by intrarectal administration of dinitrobenzene sulphonic acid (DNBS, 8 mg/mouse). CBD (1-10 mg/kg, i.p.) was injected once a day for six consecutive days starting from three days before the induction of colitis. Colonic damage was assessed by means of macroscopic and histological scores. In the inflamed colon, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression were evaluated by Western blot, nitrite levels by a fluorescent method, interleukin-1β and interleukin-10 levels by ELISA, and endocannabinoid levels by isotope-dilution liquid chromatography-mass spectrometry. Colitis significantly impaired body weight gain and increased all inflammatory parameters (including COX-2 and iNOS expression, nitrite, and interleukin-1β levels) as well as increased and reduced endocannabinoid levels and interleukin-10 levels, respectively. Pre-treatment with CBD: i) reduced colon injury (macroscopic and histological score), interleukin-1β and nitrite levels, inducible iNOS (but not COX-2) expression, and endocannabinoid changes; ii) increased interleukin-10 levels associated with DNBS administration. These results suggest that administration of CBD may be potentially beneficial for the treatment of IBD.

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Uptake and toxicity of gold nanoparticles in rat precision-cut liver slices

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Nanoparticles are structures with three external dimensions in the "nano" scale (<100 nm). Data support that they can enter the systemic circulation in function to their size (Sadauskas et al. 2009). This ability is used to drive new therapeutic compounds into specific cell targets. It cannot be excluded, however, that the nanoparticles could interfere with cellular functions and promote toxic effects (Khan et al. 2007; Goodman et al. 2004). This demands careful toxicological investigations of adverse effects of nanostructures in view of their increasing production and use. Since several studies suggest liver as a target organ of nanoparticles (Saura 2009), the aim of the present study was to evaluate the possible toxic effects of gold nanoparticles (5-10 nm) in rat precision-cut liver slices. Slices (200-300 μm) were individually incubated in RPMI 1640 under 95% O_2 5% CO_2 atmosphere at 37°C for 2-24 h in 12 well plates in the presence of increasing concentrations (5-500 μM) of gold nanoparticles, suspended in polyvinylpyrrolidone (PVP). Viability was evaluated by measuring LDH release into the medium. Tissue MTT reduction as well as GSH levels, the latter taken as an oxidative stress index, were also measured. Metabolic activities towards xenobiotics were assessed by using 7-ethoxycoumarin as a marker substrate and β-naphthoflavone or phenobarbital as CYP inducers. The nanoparticles uptake was investigated by histochemical analysis in slices incubated with 50 μM and 500 μM nanoparticles at different times (30 min-24 h) by using a Philips 201 TEM.
In liver slices the uptake of gold nanoparticles was apparent within endocytotic forming vesicles as early as 30 min and it was definitively evident after 1 h. In this case, nanoparticles were found under the plasma membrane of the most superficial hepatocytes. After 2 h and up to 24 h of incubation, gold nanoparticles formed endosome-like vesicles that were spotted deep into the cytoplasm of hepatocytes and even within cells of the second layer of the slices. The presence of gold nanoparticles was observed not only in hepatocytes but also in other liver cell populations like endothelial and Kuppfer cells.
Although the uptake of gold nanoparticles by the liver was unproblematic, no toxic effects were observed. LDH release, MTT reduction, and GSH levels of gold nanoparticles-treated slices, in fact, were not statistically different from those observed in slices incubated with PVP alone (control) under all experimental condition. Accordingly, phase I and II metabolism of 7-ethoxycoumarin were not affected while the inducibility of CYP isoenzymes was similar in control and treated slices.
In conclusion, these results indicated that the uptake of gold nanoparticles by rat liver slices did not imply an altered cellular physiology. Moreover, rat precision-cut liver slices, already described as a useful experimental model to study xenobiotic toxicity and metabolism (De Kanter et al. 2002; Tzankova et al. 2004), represent also a good approach to study toxicity of nanoparticles.

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Expression of adrenomedullin system in human thymus: an immunohystochemical study

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Thymus provides a variety of specialized microenvironment that support and direct T cell differentiation and selection (Miller et al. 1961). In a review by Takahama (2006), each of this T cell maturation events takes place in a discrete region of thymus and relies on the interaction of immature T cell with specialized thymic epithelial cell that are located in different areas of the gland (cortex, medulla, Hassall's corpuscles).
Numerous data show that thymus cell differentiation and generation of the T cell repertoire is influenced and controlled by hormones and neuropeptides that acts in a autocrine/paracrine manner.
Adrenomedullin (ADM) is an autocrine/paracrine peptide produced by several cell types, namely endothelial cells, cardiac myocytes, adrenal cortex, and thymus. ADM is produced by the post-translational proteolytic cleavage of a 185-amino acid prohormone, the prepro-ADM, a 52-amino acid peptide in human, which exerts a potent long-lasting hypotensive effect; also ADM has been show to have a remarkable range of actions, being involved in the regulation of blood pressure, vascular tone, vascular permeability, vascular regeneration, cell growth and differentiation (Nikitenko 2006; Ribatti 2005). ADM interacts with two different receptors subtypes called RAMPs (receptor activity-modifying proteins), which associate with the calcitonin receptor like receptor (CRLR) to dictate its ligand binding specificity. In particular, association of CRLR with RAMP2 or RAMP3 confers preferential ADM binding (Hinson et al. 2000).
Recent *in vitro* studies in rats suggested that ADM may play a role in thymus growth and thymocytes differentiation by controlling proliferative and apoptotic events (Belloni et al. 2003).
In this study we investigated, by immunohistochemistry staining, the expression of ADM and RAMP2 in human thymus. Human thymic fragments (5 mm thick) were obtained from patients undergoing open heart surgery in the Paediatric Cardiosurgery Division of the University of Padova and processed for immunohistochemistry (Belloni et al. 2005).
Our results indicate that human thymus stores ADM which is located mainly in thymic epithelial cells, mast cells, and blood vessels. In the cortex, the reaction can be evidenced mainly at epithelial cells level, identified with cytokeratin antibody, but not in T cells. The medulla shows a more intense reaction, due to numerous epithelial networks and to the cells of Hassal's corpuscles. Blood vessels wall (muscular and endothelial layers) of the capsular region and inside lobules, particularly at the cortico-medullary junctional level, is tipically immunostained with ADM. Immunofluorescence double reaction evidenced numerous tryptase- and ADM-positive mast cells in close association with blood vessels. RAMP2 and RAMP3 immunoreactivity was also observed in all thymuses examined. Our results indicate that the ADM system is expressed in human thymus. It can be hypothesised that ADM is involved in thymic microenvironment plasticity and reorganization, acting in an autocrine/paracrine manner or through regulation of other factors influencing such events.

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Lysozyme, a multi-role regulatory small protein, as chemopreventive drug in the diabetic nephropathy

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The present doctoral work focuses on the study of lysozyme's activity in diabetic nephropathy. Diabetic nephropathy is one of the major microvascular complications of diabetes and it is the leading cause of end-stage kidney disease in the developed countries. Chronic hyperglycemia, a typical condition of diabetes, is considered the most significant cause of diabetes complications and, in particular, its deleterious effects seem to be attributable to the formation and accumulation of sugars-derived substances called advanced glycation endproduct (AGE). AGE are a chemically heterogeneous group of macromolecules that are physiologically and non-enzymatically formed by the interactions of reducing sugars with free amino groups of proteins, lipids and nucleic acids, and their formation increases under high glucose concentration (Forbes et al. 2003). According to bibliography, hen egg white lysozyme (HEWLZ) can act as AGE's scavenger (Zheng et al. 2001) and a more recent study (Cocchietto et al. 2008) has shown that HEWLZ can significantly prevent a number of typical modifications of the early stage of diabetic nephropathy i.e. microalbuminuria and glomerular hypertrophy. Among the most evident effects of AGE there is an increased formation of intracellular reactive oxygen species (ROS) (Alikhani et al. 2007). AGE can directly lead to enhanced formation of free radicals, but their effects can also result from the interactions between AGE and their receptors, among which RAGE (receptor for AGE) is the most studied. On the basis of these results, the rationale of the research is that HEWLZ could play an intracellular role in glomerular and tubular cells in the kidney, regulating a number of pathways involved in the control of oxidative stress. The first goal of the present work is the optimization of an *in vitro* cellular system in which to simulate AGE-induced oxidative stress. Porcine kidney cell line (LLC-PK1), due to their renal origin, and adult dermal microvascular endothelial cell line (ADMEC), due to vascular involvement in the contest of diabetic complications, were chosen to verify their viability after short- and long-time exposure to AGE, using the MTT and SRB tests. The viability of both cell lines was not significantly altered after exposure, for 72 h, to different AGE concentrations (1, 10, 50, 100, and 200 ug/ml) (Cai et al. 2006) with the only exception of ADMEC, exposed to the highest treatment concentrations and assayed with MTT. Subsequently, we tested the production of intracellular ROS after cells exposure to AGE. LLC-PK1 cells ROS production seems to be not affected by AGE, while some significant increase has been seen on ADMEC. Nevertheless, a comparable increase occurs after the treatment with an equidose control of BSA. This aspect is still unclear and it will be object of further studies. On the basis of these results we can conclude that the concentrations of AGE chosen are, probably, too low to induce a significant ROS production *in vitro* on these cell lines.
The next step will be that of verifying if HEWLZ is capable to prevent ROS production or to scavenge ROS after its AGE-induced production. Considering that ROS have also a cell signalling role, the following step will be that of focussing on HEWLZ activity on signalling cascades such as NF-κB and MAPK (Hancock et al. 2001). In conclusion, the first endpoint of the present work will be that of understanding whether there is a link between the results obtained at systemic level and the intracellular effect of HEWLZ in the contest of diabetic nephropathy.

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GW0742, a high affinity PPAR-β/δ agonist, reduces lung inflammation induced by bleomycin instillation in mice

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Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors belonging to a family of ligand-activated transcription factors. Upon heterodimerization with the retinoic X receptor (RXR), PPARs bind to specific peroxisome proliferator-response elements (PPREs), located in the regulatory regions of target genes (Chinetti-Gbaguidi et al. 2009). When activated, the transcription factors exert several functions in development and metabolism (Tenenbaum et al. 2005).
There are three PPAR subtypes encoded by separate genes, showing distinct but overlapping tissue distribution, and commonly designated as PPAR-α (NR1C1), PPAR-γ (NR1C3), and PPAR-β/δ (NUC1, NR1C2), or merely –δ (Sheng et al. 2008).
PPAR β/δ is especially expressed in white adipose tissue, heart, muscle, intestine, placenta, and macrophages (Braissant et al. 1996). Recent data have clarified its metabolic roles and enhanced the potential role of this receptor as pharmacological target. Moreover, although its role in acute inflammation remains unclear, being the nuclear receptor PPAR β/δ widely expressed in many tissues, including the vascular endothelium (Piqueras et al. 2009), we assume that the infiltration of PMNs into tissues, a prominent feature in inflammation, may be also related to PPAR β/δ.
PPAR β/δ is activated by unsaturated or saturated long-chain fatty acids (Amri et al. 1995), prostacyclin, retinoic acid, and some eicosanoids (Hertz et al. 1996). Several animal studies reveal that PPAR-β/δ plays an important role in the metabolic adaptation of many tissues to environmental changes (Tenenbaum et al. 2005). It appears to be implicated in the regulation of fatty acid metabolism of skeletal muscle and adipose tissue by controlling the expression of a gene involved in fatty acid uptake, β-oxidation, and energy uncoupling (Fredenrich et al. 2005).
The aim of this study was to investigate the effects of GW0742, a synthetic high affinity PPAR β/δ agonist and its possible role in preventing the advance of inflammatory and apoptotic processes induced by bleomycin (BLEO) that long-term leads to the appearance of pulmonary fibrosis. Mice subjected to intratracheal instillation of BLEO (1 mg/kg), a glycopeptide produced by the bacterium *Streptomyces verticillus*, develop lung inflammation and injury characterized by a significant neutrophils infiltration and tissue oedema.
Our data showed that GW0742-treatment (0.3 mg/kg, 10% DMSO, i.p.) has therapeutic effects on pulmonary damage, decreasing many inflammatory and apoptotic parameters detected by measurement of: i) cytokine production; ii) leukocyte accumulation, indirectly measured as decrease of myeloperoxidase activity; iii) IκBα degradation and NF-κB nuclear translocation; iv) ERK phosphorylation; v) oxidative stress by NO formation due to iNOS expression; vi) nitrotyrosine and PAR localization; vii) the degree of apoptosis, evaluated by Bax and Bcl-2 balance, FAS ligand expression, and TUNEL staining.
Taken together, our results clearly show that GW0742 reduces the lung injury and inflammation due to the intratracheal BLEO-instillation in mice.

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A_{2A} and CB_1 receptors form heterodimers and functionally interact in human dermal fibroblasts: a new target for modulating collagen production in systemic sclerosis?

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My doctoral work has dealt with the role of both the purinergic (A_{2A} receptor) and cannabinoid system (CB_1 and CB_2 receptors) in fibroblasts of patients affect by systemic sclerosis (SSc) (Gabrielli et al. 2009).
Many data suggest that adenosine promotes the development of tissue fibrosis in different organs, via the interaction with A_{2A} receptor (Chan et al. 2006a; Chan et al. 2006b; Nakav et al. 2009); also cannabinoids seem to be able to modulate fibrogenesis (Graham et al. 2009), with an opposite effect of receptor stimulation with the CB_1 promoting and CB_2 inhibiting the fibrotic process, respectively (Caraceni et al. 2009; Michalski et al. 2008; Akhmetshina et al. 2009; Garcia-Gonzalez et al. 2009). Moreover, recent studies on central nervous system dimostrate a physical and functional interaction between A_{2A} and CB_1 receptors, resulting in a strict dependence for CB_1 signalling on A_{2A} receptor co-activation (Carriba et al. 2007; Ferré et al. 2009).
The present study, performed on dermal fibroblasts from patients with SSc and healthy volunteers, and on a human dermal fibroblasts line (HDFa cells), is aimed to: i) evaluating the extent of A_{2A} expression and its role in the regulation of collagen biosynthesis; ii) confirming the formation of A_{2A}-CB_1 heterodimers also in these cells; iii) testing the putative synergistic anti-fibrotic effect of the concomitant modulation of purinergic and cannabinoid system.
A_{2A}, and both the CB_1 and CB_2 cannabinoid receptors, are over-expressed in SSc fibroblasts, which also showed a fibrogenetic phenotype when compared with controls. A_{2A} and CB_1 receptors co-immunoprecipitate in SSc cells, in healthy cells and in HDFa cells, thereby suggesting their physical interaction as a heterodimers. Pharmacological activation of A_{2A} receptors with agonists induces a further increase in collagen production in SSc fibroblasts, which is conversely inhibited by the cell stimulation with A_{2A} receptor antagonist and a CB_1-CB_2 cannabinoid agonist.
Currently, we are studying the effect of the co-incubation of SSc fibroblasts with A_{2A} antagonist and non-selective cannabinoid agonist to evaluate if actually exist a synergistic activity of these two systems in modulating collagen production.

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The dopamine D_3 receptor agonist 7-OH-PIPAT prevents apoptosis in schwannoma cells

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Emerging evidences indicate that the dopamine D_3 receptor (D_3R) mediates protective roles both in neuronal and non-neuronal cell lines. In a previous study we have proposed that neurofibromin, a large tumor suppressor protein encoded by the neurofibromatosis type 1 gene (NF1), may increase susceptibility to apoptosis after serum deprivation in schwannoma cells, thus acting as a proapoptotic gene. In addition, it has been observed that D_3Rs are functionally correlated to neurofibromin. In this study, we examined whether 7-OH-PIPAT, a potent dopamine D_3R agonist, exerts an antiapoptotic role under the same culture conditions and then correlated this effect to changes in NF1 expression.
Results showed that serum deprivation caused a significant reduction of cell viability both after 24 and 48 h ($P<0.001$). Treatment with increasing concentrations of 7-OH-PIPAT (1 nM to 10 μM) induced a progressive increase in cell viability both after 24 h and 48 h, as compared to vehicle-treated cells, with significant changes at the highest concentrations tested (1 and 10 μM). Consistently, at the latter two concentrations, a significant reduction in oligonucleosomes formation was observed, thus suggesting an antiapoptotic role of 7-OH-PIPAT. These results were confirmed by Hoechst 33254 nuclear staining.
To investigate whether these effects were correlated to changes in NF1 transcript and protein expression, quantitative real-time PCR, Western blot, and immunofluorescence analyses were performed. Results demonstrated that the upregulation of NF1 transcripts and protein levels induced by serum withdrawal were remarkably attenuated by 1 and 10 μM agonist treatment within 24 h ($P<0.01$ and $P<0.001$, respectively), whereas similar effects were observed already at a lower concentration (100 nM) after 48 h treatment ($P<0.001$).
In conclusion, these results suggest that D_3R might mediate the protective response to serum deprivation in schwannoma cells through the inhibition of NF1 gene expression.

Growth hormone secretagogues and skeletal muscle: effect of L163,255 on Ca^{2+} homeostasis and cells viability

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The synthetic growth hormone secretagogues (GHS) and the endogenous ghrelin are small molecules proposed as pharmacological tools for the treatment of GH deficiency conditions in view of their ability to stimulate the GH release. In pituitary gland, stimulation of GH release by GHS is mediated by a G-protein-coupled specific receptor and involves Ca^{2+}-and phospholipid-dependent protein kinase C (PKC), as well as K^+ and Ca^{2+} channels (Smith 2005). Other than in pituitary gland, GHS receptor binding sites are documented in peripheral tissues accounting for a series of GHS pleiotropic effects. Accordingly, ghrelin and GHS have several cardiovascular activities including cardioprotective effects against myocardial ischemia (Cao et al. 2006).
A direct action of GHS on skeletal muscle has also been proposed, as they reduce resting Cl^- and K^+ conductances in muscle fibres, probably through the activation of a GHS-receptor linked to PLC/PKC/Ca^{2+} signalling (Pierno et al. 2003). By using fura-2 fluorescent measurements, in this study we evaluated the effect of L163,255, a non peptidyl GHS, on Ca^{2+} homeostasis of rat *extensor digitorum longus* (EDL) fibers mechanically isolated. *In vitro* application of L163,255 increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner with an IC_{50} of ~300 μM. Particularly, application of 200 μM L163,255 led to $[Ca^{2+}]_i$ increase from 26±2 nM to 164±34 nM after 10 min of incubation. Removal of external Ca^{2+} in the bath solution did not abolish L163,255 effects. On the contrary, pre-incubation with the Ca^{2+}-ATPase inhibitor thapsigargin or with the mitochondrial permeability transition pore (PTP) inhibitor cyclosporin A partially and strongly reduced L163,255-induced Ca^{2+} transient, respectively, suggesting the involvement of thapsigargin-sensitive Ca^{2+} stores and mitochondria in the drug action. Other than a key determinant of muscle contraction, Ca^{2+} is recognized to be a fundamental second messenger involved in controlling apoptotic pathways and consequently cell fate. Particularly, release of Ca^{2+} via the PTP occurs during cellular injury as well as programmed cell death (Smaili et al. 2000). Thus, the effect of L163,255 on cell viability of C2C12 myoblasts has also been examined using the tetrazolium assay. L163,255 induced cell death in a concentration-dependent manner. Particularly, at concentration of 50 μM and 100 μM, the cell number decreased by 53.0±2.6% and 96.0±1.0%. In comparison to L163,255, *in vitro* application of ghrelin at physiological concentration, produced very small effects, both on Ca^{2+} homeostasis and cell viability. Furthermore, preliminary results obtained by using the synthetic peptidyl GHS hexarelin suggested that drug activity on skeletal muscle is strictly dependent on the nonpeptidyl structure.
Taken together, our results reveal that GHS could significantly interfere with skeletal muscle functionality. This novel GHS-mediated mechanism could significantly contribute to elucidate how in skeletal muscle the Ca^{2+} signalling machinery is connected to the cellular processes leading to cell death as well as to design more specific drugs with peripheral or central activity.

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Protective effect of TAT-parkin on neuronal cells

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Parkinson's disease (PD) is a neurodegenerative disorder-caused by dopaminergic (DN) death in the *substantia nigra pars compacta* (SNpc). This and the absence of Lewy bodies are the pathological hallmarks of autosomal recessive juvenile parkinsonism, which is known to be caused by a loss-of-function mutation in the *parkin* gene (Yang et al. 2005). Parkin protein is an E3 ubiquitin-protein ligase that polyubiquitinates abnormal proteins (Shimura et al. 2000). Several recent studies have attributed a protective role of parkin in DN survival against a variety of toxic stimuli (Staropoli et al. 2003). Here we have investigate the neuroprotective effect of parkin against 6-hydroxydopamine (6-OHDA)-induced neurodegeneration using rat adrenal pheochromocytoma PC12 cells, either naive or induced to develop some of the phenotypic traits of DN neurons by exposure to nerve growth factor (NGF, 50 ng/ml). Human parkin was fused to a protein transduction domain derived from the human immunodeficiency HIV TAT protein (TAT-parkin) to facilitate passage across the plasma cell membrane. Protein purification was carried out under denaturing conditions using a NTA-Ni^{2+} column in the presence of 8 M urea (pH 4.5), 250 mM imidazole followed by a gel filtration G25 column. To examine response to oxidative stress, PC12 cells were treated with 5-300 μM 6-OHDA. This resulted in a time- and concentration-dependent decrease in cell viability (40% at 0 h and 70% at 6 h), with an estimated DL_{50} of 50 μM 6-OHDA. PC12 cells, pre-incubated with 100 μM TAT-parkin (a non-toxic concentration) for 24 h, were protected against 6-OHDA (50 μM) toxicity only at 0 h. PC12 cells were then pre-incubated with 100 μM TAT-parkin for 24 h, before treatment with 50 μM 6-OHDA and then re-incubated with 100 μM TAT-parkin: this treatment regimen increased survival at all incubation times. These results suggest that TAT-parkin is able to protect PC12 cells against cytotoxicity by 6-OHDA only if present in the culture medium before, during, and after treatment with 6-OHDA. To further assess the pro-survival action of parkin, we used a system where parkin is endogenously expressed: PC12 cells were transfected with either PCDNA3-parkin (wild-type, WT) or PCDNA3-mutant R42P parkin. Non-transfected PC12 cells were used as control. All cells were treated with 50 μM 6-OHDA for 2, 4, 6, 12, and 24 h followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell viability decreased in a time-dependent manner in all cell clones and untransfected PC12 cells, while PC12 cells over-expressing WT parkin were more resistant to 6-OHDA-induced oxidative stress. We next examined 6-OHDA generation of reactive oxygen species in these cells, using the fluorescent probe 2',7'-dichlorofluorescein diacetate. Also in this case the production of free radicals increased in a time-dependent manner (from 2 to 24 h), while cell viability concomitantly decreased during this time. Over-expression of parkin protected PC12 cells from intracellular oxidant stress. In fact, MTT reduction decreased significantly only after 12 h of treatment with 6-OHDA, while cell viability in the other clones decreased already after 2 h. Abnormalities in the ubiquitin-proteasome system and the autophagy-lysosome pathway are involved in the development of PD (Yang et al. 2009). When the same clones were treated with either a selective proteasome inhibitor MG132 (2.5 μM), or a a macroautophagy inhibitor 3-methyladenine (10 mM) for 12 h, improved survival was again evident in parkin over-expressing cells. Our data suggest that parkin, presented as a TAT-fusion protein, is able to protect DN PC12 cells against the toxic damage of 6-OHDA, MG132, and 3-methyladenine, and propose that parkin represents a potential new therapeutic target in PD.

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Deregulation of calcium homeostasis in a novel model of Alzheimer's disease

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Alzheimer's disease (AD) is the most common age-related neurological disorder and is one of the most devastating diagnoses that patients can receive. The disease is characterized by progressive loss of memory, cognitive impairment and dementia leading eventually to death. Histological hallmark of the disease is the extracellular deposition of plaques of amyloid β (Aβ) in different regions of the brain, especially in hippocampus. The Aβ is produced by cleavage of the amyloid precursor protein (APP), which is processed by concerted action of two secretases, β-secretase (BACE) and γ-secretase. The third protease which processes APP, ADAM10 (a disintegrin and metalloproteinase 10), is an α-secretase which is involved in the physiological non-amyloidogenic APP processing. The beneficial activity of ADAM10 is mediated by interaction with the synapse-associated protein-97 (SAP97), which mediates ADAM10 trafficking and promotes its activity (Marcello et al. 2007).
Deregulation of Ca^{2+} homeostasis, especially at the synaptic level, plays an important role in pathogenesis of AD (Berridge et al. 2010; Supnet et al. 2010). Moreover, the abnormalities of the glutamatergic synaptic signalling occur early in the course of the disease, which is reflected in a loss of the synaptic plasticity (Gylys et al. 2004).
In this regard, a novel model of AD has been developed in which the interaction ADAM10/SAP97 is disrupted using a cell-penetrating Tat-peptide (Green et al. 1998) fused to ADAM10 proline-rich domains (Tat-Pro $ADAM10^{709\text{-}729}$). In mice injected i.p. with 3 nmol/g Tat-Pro $ADAM10^{709\text{-}729}$ decrease of soluble APPα (sAPPα, a product of ADAM10 cleavage), but not total sAPP, was detected already 24 h after treatment.
In this context, we were interested in investigating alterations of the synaptic Ca^{2+} signalling in primary cultures of rat hippocampal neurons treated with Tat-Pro $ADAM10^{709\text{-}729}$ peptide. Employing real-time PCR we have analyzed levels of transcripts of the key components of Ca^{2+} signalling such as the group I metabotropic glutamate receptors (mGluR1 and mGluR5), the ionotropic glutamate receptors (NMDA) and the neuronal type IP_3 receptor (IP_3R1). We found that the levels of mRNA of these genes were significantly increased in neurons treated with Tat-Pro $ADAM10^{709\text{-}729}$. Notably, the IP_3R1 was up-regulated also at the protein level as measured by Western blot analysis, suggesting enhanced Ca^{2+} signalling via mGluR1/5. To investigate whether the gene expression is the cause of deregulated synaptic Ca^{2+} homeostasis, the work is underway to analyze Ca^{2+} signals induced by glutamate, NMDA and (S)-3,5-dihydroxyphenylglycine (DHPG) in neurons treated with Tat-Pro $ADAM10^{709\text{-}729}$. For measurements of Ca^{2+} transients we used both the fura-2 single cell Ca^{2+} imaging and the Ca^{2+}-sensitive photo-protein aequorin which allows monitoring Ca^{2+} in chosen sub-cellular compartments such as synaptic terminals. Our results suggest potentiation of Ca^{2+} signalling in Tat-Pro $ADAM10^{709\text{-}729}$-treated neurons. Preliminary results also indicate a possible involvement of the Ca^{2+}-dependent phosphatase calcineurin in transcriptional deregulation of Ca^{2+}-related genes in Tat-Pro $ADAM10^{709\text{-}729}$ model. Our future plans also foresee comprehensive microarray analysis aiming of search of Ca^{2+} signalling-related genes whose expression may change in Tat-Pro $ADAM10^{709\text{-}729}$ model of the AD.
Taken together, our results are in line with the "Ca^{2+} deregulation" hypothesis of the AD (Berridge et al. 2010; Supnet et al. 2010) and demonstrate that Tat-Pro $ADAM10^{709\text{-}729}$ model can be used for investigation of AD-related deregulation of synaptic Ca^{2+} homeostasis.

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Lithocholic acid is a competitive and reversible EphA2-ephrinA1 antagonist

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Eph-ephrin system plays a central role in a large variety of human cancers. In fact, upregulated expression and/or de-regulated function of Eph-ephrin system promotes tumorigenesis and development of a more aggressive and metastatic tumour phenotype. In particular EphA2 upregulation is correlated with tumour stage and progression and the expression of EphA2 in non-transformed cells induces malignant transformation and confers tumorigenic potential (Zelinski et al. 2001; Miyazaki et al. 2003; Kinch et al. 2003; Herrem et al. 2005). The aim of my work was to identify and characterize small molecules able to modulate EphA2-ephrinA1 activity. We performed an ELISA-binding assay screening on a chemical library including different drugs and endogenous bioactive molecules in order to identify scaffolds that might be utilized to design chemical entities able to inhibit the interaction between EphA2 extracellular domain and ephrinA1. In this screening we detected lithocholic acid (LCA) as a competitive and reversible ligand of EphA2-ephrinA1 binding behaving a K_i=49.0 μM and a Hill coefficient of 0.86. LCA was unable to discriminate different Eph kinase classes suggesting an interaction with a highly conserved region essential for both Eph A and B kinase binding to their physiological ligands. These evidences could suggest an interference of LCA with the proper full insertion of the G-H loop of the ephrin ligand into the Eph-kinase hydrophobic channel. Anyway, structural studies will be essential to clarify the dynamic of interaction.
Functional studies were performed in cultured cells to evaluate agonist or antagonist properties of LCA and other bile acids at Eph receptors using PC3 prostate adenocarcinoma cells and HT29 colon adenocarcinoma cells as a model for their known ability to naturally express EphA2 (Miao et al. 2000; Tanaka et al. 2005). In these studies EphA2 phosphorylation was induced with 0.25 μg/ml ephrinA1-Fc, in presence or absence of bile acids and using 10 μM Dasatinib as reference compound (Chang et al. 2008). Consistently with binding studies 100 μM cholic, deoxycholic, and chenodeoxycholic acids were inactive whereas LCA dose dependently antagonized Eph-kinases phosphorylation induced by ephrin at no-cytotoxic concentrations, evaluated using the MTT colorimetric assay.
On the other hand, LCA resulted to be inactive towards EGFR phosphorylation induced by EGF supporting the specificity of LCA interaction.
Because of LCA plays a role in colon cancer development but the molecular mechanism involved is far to be clear, our findings could be useful for further works aimed at the study of intestinal cell renewal. Moreover, it could be a starting point for the development of synthetic molecules able to treat pathologies where Eph-kinases are involved, such as pathological angiogenesis, cancer and nerve injury.

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Epigallocatechin-3-gallate (EGCG), the most abundant polyphenolic compound of green tea, exerts anti- or pro-oxidant activities in endothelial cells depending on pre-existing redox conditions

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Unbalanced cell redox activity and subsequent oxidative stress has been consistently demonstrated in the pathophysiology of vascular complications for metabolic or hemodynamic diseases. In the last years, increasing attention has been focused on the anti-oxidant ability of the so-called functional foods such as green tea, dark chocolate and red wine. Pharmacological characterization of bioactive compounds is paramount to understand when and why beneficial or detrimental effects may be expected. Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenolic compound contained in green tea. In diabetic and insulin-resistance conditions, EGCG improves cardiac and vascular function by stimulating the production of endothelial NO via signalling pathways involving H_2O_2, Fyn, PI 3-kinase, Akt, and eNOS (Kim et al. 2007; Potenza et al. 2007). Interestingly, few recent reports suggest that, despite its anti-oxidant potential, EGCG may increase production of reactive oxygen species (ROS) under certain conditions (Stangl et al. 2007). We hypothesized that EGCG may exert anti- or pro-oxidant activities depending on previous cellular redox state, and investigated the molecular mechanisms involved in EGCG-mediated modulation of redox balance in endothelial cells under healthy or pro-oxidant conditions. Bovine aortic endothelial cells (BAEC, Lonza) in primary culture were used at 4-6 passages. EGCG time-course (30 min – 48 h) and concentration-response (1-100 μM) experiments were carried out in BAEC under normal or oxidative stress conditions. Oxidative stress was induced by culturing BAEC under high glucose (HG; 55 mM/48 h) or insulin resistance mimicking state [100 nM insulin plus 100 μM wortmannin (IW) for 24 h]. Total and phosphorylated levels of eNOS, AMPK, and LKB1 were analyzed by immunoblotting in lysates of BAEC untreated or treated with EGCG in the absence or presence of the AMPK inhibitor Compound C (CC, 20 μM/2 h). For ROS detection, the fluorescent probes 5- and 6-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H2DCF-DA; 5 μM) and dihydroethidium (DHE; 3μM) were used to measure intracellular H_2O_2 and $O_2^{-\cdot}$ levels, respectively. Images were visualized by epifluorescent microscope with appropriate filters. In control BAEC, acute stimulation with EGCG increased eNOS, AMPK, and LKB1 phosphorylation levels in a short term, whereas prolonged stimulation resulted in increased production of both H_2O_2 and $O_2^{-\cdot}$ and impaired cell survival. Thus, in endothelial cells under basal conditions, EGCG has beneficial effects in acute stimulation, and detrimental effects in the long term stimulation. In HG- or IW-treated BAEC, nuclear $O_2^{-\cdot}$ and cytoplasmic H_2O_2 concentrations were significantly increased over basal levels. Interestingly, in HG- or IW-treated BAEC, stimulation with EGCG significantly decreased nuclear $O_2^{-\cdot}$ to levels comparable with those obtained in BAEC pre-treated with anti-oxidants such as superoxide ndismutase (180 U, 30 min), apocynin (100 μM, 30 min), or catalase (500 U, 30 min). Catalase administration did not further reduce ROS levels in IW-BAEC pre-treated with EGCG. Importantly, inhibition of AMPK activity by CC (20 μM, 2 h) significantly blunted EGCG-dependent reduction of ROS in IW-treated BAEC. Results obtained so far confirm that EGCG has a dual effect on cellular redox balance of endothelial cells: while its anti-oxidant effect prevails in pre-existing oxidative stress, EGCG may act as pro-oxidant under basal conditions. Future studies will analyze the signalling molecules involved in EGCG-dependent regulation of ROS production in endothelial cells.

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Role of apocynin, an inhibitor of NADPH oxidase, in the inflammatory process induced by an experimental model of spinal cord injury

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The importance of reactive oxygen species in spinal cord injury is supported by the large number of experimental and clinical studies demonstrating potential neuronal efficacy of agents with anti-oxidant proprieties (Genovese et al. 2005). NADPH-oxidase is an enzyme responsible for free radicals production, and inhibition of this enzyme represents an attractive therapeutic target for the treatment of many diseases. To counteract oxidative stress, the body produces an armoury of antioxidants to defend itself, which however are sometimes insufficient to effectively defend the organism from ROS (Luchtefeld et al. 2008). There are a lot of substances that have been researched in order to find a way to inhibit production of ROS, and thus protect the body from diseases.
Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of the Himalayan herb *Picrorhiza kurrooa* Royle (*Scrophulariaceae*) that is well known in traditional Indian medicine (Ayurveda). It is an acetophenone to which a range of biological activities is attributed (Hougee et al. 2006). The structure of NADPH-oxidase is quite complex, consisting of two membrane-bounded elements (gp91phox or Nox 2 and p22phox), three cytosolic components (p67phox, p47phox, and p40phox), and a low-molecular-weight G protein (either rac 2 or rac 1). The generation of superoxide anions by NADPH oxidase serves as a host defense mechanism against invading microorganism infection and the enzyme is present in phagocytic cells, such as monocytes and neutrophils (Dorman et al. 2006). Recent studies have also suggested that NADPH oxidase is expressed in the central nervous system in neurons, astrocytes, and microglia (Bedard et al. 2007). Apocynin is an inhibitor of the intracellular translocation of two critical cytosolic components of the NADPH-oxidase complex present in the cell membrane.
The aim of this study was to investigate the effects of apocynin, in the modulation of secondary injury in the spinal cord. The injury was induced by application of vascular clips (force of 24 g) to the *dura* via a four-level T5-T8 laminectomy in mice. Treatment with apocynin (5 mg/kg, 10% DMSO) 1 and 6 h after the trauma significantly decreased: i) the degree of spinal cord inflammation and tissue injury (histological score); ii) neutrophil infiltration (myeloperoxidase activity); iii) intercellular adhesion molecule (ICAM-1) and platelet-adhesion molecule expression (P-selectin); iv) NF-κB expression; v) nitrotyrosine and poly-ADP-ribose formation; vi) pro-inflammatory cytokines production (TNF-α and IL-1β); vii) apoptosis (TUNEL staining, FAS-ligand expression, Bax and Bcl-2 expression); viii) mitogen-activated protein kinase (MAPK) activation (P-38 and SAPK-JNK). Moreover, apocynin significantly ameliorated the loss of limb function (evaluated by motor recovery score).
Thus, we propose that NADPH oxidase inhibitors may be useful in the treatment of inflammation and tissue injury associated with spinal cord trauma.

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Stable isotope tracers to estimate surfactant metabolism in preterm infants with respiratory distress syndrome

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Pulmonary surfactant is a phospholipid-protein complex that lines the inner surface of the lung and is essential for normal pulmonary function. Surfactant, synthesized by lung type II cells, acts to promote lung stability by reducing surface tension, while also protecting against inhaled pathogens. Surfactant is composed of 90% phospholipids (PL) and 10% proteins by weight. A specific PL, disaturated phospatidylcholine, is predominantly responsible for the modulation of surface tension at the alveolar air-liquid interface. Surfactant deficiency is the hallmark of respiratory distress syndrome (RDS), the most common respiratory disease of preterm infants: the lung function is impaired and the surfactant quantity and composition, included DSPC, is reduced. Systemic and pulmonary inflammation markers are involved in these pathways; in particular phospholipase A_2 is responsible of the hydrolysis of the ester bonds at the sn-2 position of membrane PL. In the lung macrophages and type II cells, secreted on the alveolar surface and activated by inflammatory mediators, produce this enzyme that catabolises surfactant PL yielding lyso PL and free fatty acid, which in turn induce further lung injury. In the early 90's administration of exogenous surfactant was introduced as a routine treatment greatly reducing morbidity and mortality in preterm infants with RDS (Jobe et al. 1993). Recently, stable isotopes have been used to investigate surfactant synthesis and turnover *in vivo* in infants with lung injury. During the last 15 years our research group developed new safe and ethically acceptable methods based on stable isotope technology, suitable for the study of both endogenous and exogenous surfactant metabolism in humans. Molecules labelled with stable isotopes were used either as metabolic precursors of surfactant dipalmitoyl phosphatidylcholine (DPPC) or as a preformed DPPC tracer, in order to measure the tracer precursors incorporation rate or the tracer turnover, respectively. These studies show that plasma free fatty acids, plasma glucose, and body water are all suitable metabolic precursors for DPPC synthesis and that DPPC showed increased catabolism due to different pulmonary disease (Cogo et al. 2003; 1999; Torresin et al. 2000; Bunt et al. 1998). My PhD project was initially focused on the most suitable metabolic substrate to measure surfactant DPPC synthesis focusing on the difference between incorporation of the plasma palmitate fatty acid precursors vs de novo lipogenesis palmitate incorporation; in the second and third years we investigated the turnover of $^2H_3$-$U^{13}C$ DPPC, a newly synthesized stable isotope tracer, to discriminate kinetics of the sn-1 and sn-2 PA moieties in preterm infant with RDS. We studied preterm infants who required a single or multiple doses of exogenous surfactant and prolonged mechanical ventilation for RDS. In the first study, infants were divided into 3 groups due to the simultaneously administration of two DPPC precursors differently labelled ($^2H_3$PA, $U^{13}C$-PA, ^2H_2O, $U^{13}C$ glucose) in order to trace the *de novo* (glucose or water) and the plasma palmitic free fatty acid (PA-FFA) pathways. In the second study, infants received exogenous surfactant mixed with $^2H_3$-$U^{13}C$ DPPC. We collected sequential tracheal aspirates and plasma samples, we extracted FFA-PA and PA-DPPC, isolated by TLC, and their isotopic enrichments were measured by GC/MS. The kinetics parameters evaluated for each tracer were: secretion time, fractional synthetic rate, peak time, and half-life. The first study showed that while glucose and water are able to trace the contribution of the *de novo* PA-DPPC synthesis pathway, plasma FFA-PA evaluates the contribution of circulating PA to DPPC synthesis; we also showed, by between- and within-group analyses, that the lipogenic pathway and the incorporation of plasma PA contributed almost equally to surfactant synthesis (Cogo et al. 2009). Preliminary results of the second study showed that mean of $^2H_3$-PA and $U^{13}C$-PA DPPC half-life were almost equal. To deeply investigate the different kinetic rate between the sn-1 and sn-2 position of the DPPC, half-life ratios $U^{13}C/^2H_3$ were calculated for all infants. Comparisons between the different pulmonary status indicate that the more severe respiratory disease tended to have a ratio less than 1, i.e. half-life of the sn-2 was shorter than half-life of sn-1. Further studies are in progress to assess if the accelerated kinetic of the sn-2 position is due to an increased inflammatory damage induced by phospholipases, or an alteration of pulmonary surfactant caused by inflammatory lipid mediators.

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Effects caused by glucosinolates on antioxidant and xenobiotic-metabolising enzymes in primary rat hepatocytes cultures

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Glucosinolates (GLs) have recently attracted intense research because of their protective attributes against cardiovascular disease and some kind of cancer. They are present in sixteen families of dicotyledonous angiosperms including a large number of edible species. GLs are β-thioglucoside-N-hydroxysulfates with a variable side chain (R) and a sulfur-linked β-D-glucopyranose moiety. Experimental data indicates that certain vegetables may offer an indirect protection against oxidative stress by activating endogenous cellular defense systems at genetic and protein levels. GLs have shown a low bioactivity as such, but once converted by myrosinase enzyme (also present in intestinal flora), they originate isothiocyanates (ITCs) (Fahey et al. 1997), which induce antioxidant enzymes through the activation of nuclear factor E2-related protein (Nrf2) receptor.

In the present study, by using primary rat hepatocytes, we investigated the effects of four glucosinolates: glucosinalbin, sinigrin, glucoiberina, and gluconasturtin on the antioxidant and drug-metabolising enzymes at transcriptional, catalytic, and immunoblotting levels. The isolation of hepatocytes was made with the method described by De Smet et al. (1998) using two layers of rat-tail collagene (type 1). Cells were treated with the myrosinase-hydrolysed GLs at doses up to 40 μM and analysed by PCR for the expression of NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1 genes. On the other hand, in the hepatocyte microsomes and 100,000 x g supernatants, prepared in the standard way, it was investigated the enzymatic activity of NAD(P)H:quinone oxidoreductase-1, glutathione-S-transferase, catalase, heme oxygenase-1, GSSG-reductase, and ethoxycumarin-O-deetylase (a marker of several CYPs). Additionally, the activation of Nrf2 was verified in cytosol and nuclei by Western blotting analysis.

All the analyzed GLs were able to activate, in a dose dependent manner, the transcription of NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1 genes and the corresponding activities. In addition, it was also observed a significant induction of glutathione-S-transferase, catalase, and GSSG-reductase antioxidant activities. On the other hand, the CYP-dependent ethoxycumarin-O-deetylase activity was decreased by sinigrin at the highest dose suggesting their cytotoxicity. In contrast, the treatment with glucosinalbin produced a dose dependent induction of this CYP activity, possibly due to the presence of an aromatic moiety in its structure. By Western blotting analysis it was ascertained that most of cytosolic Nrf2 was translocated into the nuclei after the GLs treatments indicating that the induction of the above mentioned antioxidant enzymatic activities was regulated by this transcriptional factor. In conclusion, it was found that these GLs might have a protective potential against oxidative stress similar to that shown by the classic sulforaphane. *In vivo* experiments are needed to ascertain this possibility.

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Fullerene derivatives as carriers for anticancer drugs

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Chemotherapy treatment is limited by the ability of cancer cells to become resistant to different drugs at the same time, a trait known as multidrug resistance (MDR). The mechanisms of drug resistance can develop at many levels, including alteration of the target protein, decreased membrane permeability and drug metabolism, increased processing of drug induced damage, or evasion of apoptosis (Longeli et al. 2005; Gottesman et al. 2002). The strategies such as targeted drug delivery systems have been attempted to overcome the MDR of cancer cells. Target drug delivery is the effective approach to provide the therapeutic concentration of anticancer drugs to the target cancer cells rather than the noncancerous cells by a variety of functionalized drug carriers, which thus improve the therapeutic efficacy specifically for tumour tissue (Vasir et al. 2005). Nanotechnology has the potential to revolutionize cancer diagnosis and therapy. A wide variety of different nanomaterials based on allotropic forms of carbon are currently being explored towards different applications in cancer chemotherapy. Derivatized fullerenes could be used in biomedical application and be suitable vectors for drug delivery due to their small size, large surface area and solubility (Zhanga et al. 2009). Cytotoxicity studies on different fullerenes, the process of how cells recognize fullerenes, and the mechanism of cellular uptake are the subject of this work.
The different fullerene derivatives used in this study have been synthesized exploiting the 1,3-dipolar cycloaddition of azomethine ylides to the fullerene (C60) skeleton (Maggini et al. 1993). This a very versatile reaction that allows the modification of C60 in order to render it water-soluble and to offer an anchor point for the attachment of other moieties. Thus, we have functionalized C60, and, with subsequent modifications, we have obtained compounds 1, 2, and 3. The cytotoxicity of these three fullerenes was tested on the human mammary carcinoma cell line MCF7 and on its multidrug resistant MCF7/ADR. The choice of these cells is based on our objective to conjugate the anticancer drug adriamycin to the fullerene vector. Incubation of these cells with concentrations of the three fullerenes up to 25 μM for 24, 48, and 72 h showed variable modifications of cell viability, as determined by the MTT test. In detail, compounds 1 and 3 showed only a weak or null reduction of cell viability (max 10% reduction vs untreated controls), whereas compound 2 showed a more pronounced cytotoxicity, affecting cell growth by more than 50% at 25 μM, after 72 h incubation. On this basis we decided to continue the evaluation of the biological interactions of these fullerenes with MCF7 cells using compound 1 which, among the 3 compounds tested, was the least toxic for these cells. A further test, to confirm the absence of cytotoxicity of compound 1 for MCF7 cells, was performed using the DiOC6 probe and propidium iodide (PI) by a flow cytometry approach. The combined use of PI and the DiOC6 probes further confirmed the absence of cytotoxicity of the tested compound for these two cell lines. In particular the use of DiOC6 allows us to exclude the induction of apoptotic effects by compound 1 since no alterations of the mitochondrial transmembrane potential were observed. A further analysis was done with compound 1 conjugated with FITC (fullerene 4), to study the capacity of this fullerene to bind to and to enter the MCF7 and MCF7/ADR cells. Fullerene 4 rapidly binds to both cell lines (a plateau is reached within few minutes), and it enters the cells; in fact, data clearly show a shift towards higher values of fluorescence when compound 4 is added to the cells for 48 h. Indication that the compound has entered the cells is given by the fact that there is no quenching effect with trypan blue.
In conclusion this work shows that fullerenes might be used to deliver chemicals into target cells. The example we have provided here is the conjugation between fullerene 1 and fluorescein isothiocyanate. Another important conclusion from this preliminary study is that even small modifications on the basic structure of the fullerene moiety can lead to important modifications of the biological behaviour of the resulting compound. In the present study, for example, a modest change of the molecule markedly increased cell cytotoxicity. These results stress the need to carefully test each individual compound to check the nature of its interactions with living cells.

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Nonpeptide PKRs antagonist in animal models of inflammatory and neuropathic pain

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My first year doctoral work has dealt with *in vivo* pharmacological characterization of a new prokineticin receptors antagonist, PC1. Bv8 (an amphibian secreted protein) and prokineticins (PK1 and PK2-Bv8's mammalian homologues) are a new family of chemokines identified a decade ago and linked to several biological effects like gut motility, angiogenesis, hematopoiesis, circadian rhythm, pain, and inflammation. In rodents, the activation of PKRs (PKR1 and PKR2) by Bv8 decreases the nociceptive threshold to the painful stimuli (Negri et al. 2002). Recently, it was demonstrated by our group that PK2 is over-expressed in inflammatory granulocytes, playing an important role in inflammatory pain development (Giannini et al. 2009). The triazine compound, PC1, a PKR1 preferring ligand (Balboni et al. 2008) selectively antagonizes the Bv8-induced hyperalgesia and abolishes the CFA induced thermal hyperalgesia for ~2 h in rodents. Therefore, the aim of my work was: i) to evaluate the role of PC1 chronic administration in the mouse model of inflammatory pain - intraplantar injection of CFA (20 μl per paw) produces an inflammatory reaction with a concomitant swelling of the paw and hypernociception; ii) to evaluate the role of the PK/PKRs in an animal model of neuropathic pain [chronic constriction injury (CCI)] knowing that prokineticins and their receptors are constitutively expressed in neurons, microglia, and astrocytes (Koyoma et al. 2006) and the CCI model combines nerve compression with an epineurial inflammatory lesion. The CCI model involves placement of three loose silk sutures thread ligatures on the sciatic nerve (Bennett et al. 1988). After surgery all mice (n=15) developed thermal hyperalgesia from day 3 and mechanical allodynia from day 17 on the lesion side, while sham-operated animals (n=5) did not. PC1 was administrated systemically (150 μg/kg, s.c.) twice a day for 3 days in CFA model and 4 days in CCI model. In the CFA model the mice (n=5) received PC1 on day 1 (6 h after CFA administration) up to the 4th day. In the CCI model PC1 was administered systemically to different groups of mice at different time: group 1 (n=5) received PC1 from day 3 (when thermal hyperalgesia peaked) to day 6, and group 2 (n=5) from day 17 (when allodynia peaked) to day 20 after CCI. For both models, thermal hyperalgesia (Plantar Test) and mechanical allodynia (von Frey hairs) were assessed before and for 3 hours after PC1 acute administration. After the treatment, the tests were assessed daily up to the 10th day in the CFA model and up to the 42nd day in the CCI model. To evaluate paw oedema elicited by CFA, paw volume of all tested mice (n=10) was measured daily with a plethysmometer. In the CFA model acute administration of PC1 abolished the inflammation-induced thermal and mechanical hypernociception for ~2 h. Repeated administration of PC1 reduced thermal and mechanical hyperalgesia and, also, reduced the paw oedema and accelerated the recovery to normal paw volume of treated mice compared with those treated with saline (n=5). In the CCI model, acute administration of PC1 abolished thermal hyperalgesia and mechanical allodynia for ~2 h after treatment in both groups compared to the saline treated group (n=5). Repeated administration of PC1 significantly reduced thermal hyperalgesia after the treatment, and the mechanical allodynia did not develop in group 1. In group 2, PC1 chronic administration reduced the thermal hyperalgesia and mechanical allodynia after the treatment compared with the saline-treated group. In contrast, thermal hyperalgesia and mechanical allodynia persisted in saline-treated group of CCI mice for almost 40 days. In agreement with the results observed in these two models of pain, I can conclude that the PKRs are potential targets for novel analgesic drugs.

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Leptin: a target for Alzheimer's disease?

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Leptin, the product of the *ob* gene, is an adipocyte-derived hormone with the main function in regulation of energy expenditure and food intake, due to its actions on the arcuate nucleus of the hypothalamus. In addiction to its roles in feeding and homeostatic energy control, leptin is now known to exert significant effects on reproduction, thermogenesis and evidence is growing that leptin has widespread actions in the central nervous system (CNS; Harvey 2010). Leptin mRNA and protein have been detected in several brain regions, including the hippocampus, and recent studies have shown leptin production by the brain itself. Moreover leptin markedly influences the cellular events underlying hippocampal-dependent learning and memory: leptin indeed facilitates hippocampal long term potentiation and promotes rapid changes in hippocampal dendritic morphology and synaptic density (Moult 2010). Recent studies also provide support for a link between impairment and/or altered leptin function and the development of Alzheimer's disease (AD): leptin has been shown to reduce the amount of extracellular amyloid β, both in cell culture and animal models, as well as to reduce tau phosphorylation in neuronal cells. Chronic administration of leptin resulted in a significant improvement in the cognitive performance of transgenic animal models (Greco 2010). It is speculated that a deficiency in leptin may contribute to systemic and CNS abnormalities leading to disease progression. Furthermore, a leptin deficiency may aggravate insulin-controlled pathways, known to be aberrant in AD. All these observations suggest that a leptin replacement therapy may be beneficial for these patients (Tezapsidis 2010). There are, however, still numerous questions, regarding the molecular mechanism by which leptin acts, that remain unanswered.
In order to investigate and elucidate the potential key-role of leptin in AD, my project has been divided in studies *in vitro*, *in vivo*, and in human.
In particular, we investigate the mechanism by which leptin acts with ApoE4. ApoE4 is an allelic variant of apolipoprotein E and it is well known as the major genetic risk factor for sporadic AD (Mahley 2000): 80% of patients with AD possess at least one apoE4 allele. Apolipoprotein E plays a fundamental role in the maintenance and repair of neurons. In human three isoforms exist (E2, E3, and E4). These isoforms differ in their abilities to accomplish their critical tasks and ApoE4 is associated with a wide variety of neuropathological processes. Through its lipid transport function, apoE is an important factor in repairing and maintaining synapto-dendritic connections. Impaired cognition in non-dement individuals carrying the apoE4 allele worsens with age, suggesting a global detrimental effect on the CNS (Deary 2002). Although apoE4 is strongly linked to AD pathology, its mode of action is unknown.
In the last years my group developed a potential animal model for sporadic AD that could have a more natural onset of the pathology: *apoE4*, *apoE3* Target Replacement model mice, grown in different life style conditions such as a normal diet, high saturated fats and cholesterol intake, and high carbohydrate intake. In fact, the combination of life style risk factors, as diet, and the major genetic risk factor, as ApoE4 genotype, has been proposed as main cause of sporadic AD. On this animal model we performed behavioural studies to evaluate cognition and memory impairments; also leptin expression has been evaluated *post mortem* in frontal cortex and hippocampus, cerebral areas mostly involved in AD. Meanwhile the same molecular experiments were carried on human neuronal cells and glial cells treated with apoE4, in order to evaluate the different expression of leptin after the treatment. Finally, leptin levels have been studied in human brains and cerebrospinalfluid of AD patients, both apoE4 and apoE3 carriers.
The combination of high throughput methods with the classical biochemical assays, *in vivo*, *in vitro*, and in human, will allow a deep understanding of the molecular mechanisms behind leptin and ApoE4, potential targets for the cure of AD. The experimental design has been carried on in collaboration with the Alzheimer`s Research Center of the Karolinska Institutet in Stockholm.

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Synaptic localization and activity of ADAM10 regulate excitatory synapses through N-cadherin cleavage

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N-cadherin, the most relevant cadherin in neurons, has an important role during dendrite arborisation, axon guidance and synaptogenesis. At synaptic sites, N-Cadherin is involved in the regulation of synaptic adhesion, morphology and plasticity. Recent studies have shown that N-cadherin can be cleaved by the metalloproteinase ADAM10. Here we demonstrate that inhibition of ADAM10 localization and activity to synaptic sites, by disrupting its interaction with SAP97, results in decreased ADAM10-mediated N-cadherin processing. This indicates that N-cadherin cleavage by ADAM10 occurs at synaptic sites. The consequent accumulation of the full-length form of N-cadherin is correlated to an increase in spine head width and to a modification of the number and function of glutamate receptors of AMPA-type at hippocampal synapses both *in vitro* and *in vivo*. Our results indicate that ADAM10 localization and activity at synaptic sites control the structure and function of glutamatergic synapses by regulated cleavage of N-cadherin. In particular, we demonstrate a key role for ADAM10 in the complex and coordinated sequence of events through which N-cadherin affects spine maturation.

Alterations of iNOS expression and adenosine metabolism in vascular smooth muscle cells from diabetic rats

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Vascular smooth muscle cells (VSMCs) use multiple mechanisms to generate extracellular adenosine, an important endogenous modulator that controls a variety of pathophysiological events. By interacting with P1 receptors, adenosine exerts several vasoprotective actions including inhibition of VSMC growth and stimulation of nitric oxide (NO) release from these cells (Dubey et al. 2001). Increased inducible NO synthase (iNOS) production by inflammatory cytokines is involved in diabetic vascular dysfunction (Nagareddy et al. 2009). To investigate a potential anti-inflammatory role of adenosine in this process, we determined its influence on iNOS expression in VSMCs from diabetic as compared to normoglycaemic rats. We also investigated the pathways that are responsible for extracellular adenosine elimination under the same conditions. Diabetes was induced in Sprague-Dawley rats by intravenous injection of streptozotocin 4 weeks before sacrifice. VSMCs from normal and diabetic rat aortas were incubated for 24 h in the presence of LPS (1 μg/ml) combined with a cytokine mixture comprising IL-1β (10 ng/ml), TNF-α (25 ng/ml) and INF-γ (10 ng/ml). The levels of iNOS protein were assessed by Western blotting. Adenosine, AMP, and their metabolites in the culture medium were measured by HPLC (Giron et al. 2008). Incubation of VSMCs from normoglycaemic or diabetic rats with LPS plus cytokine mixture for 24 h induced expression of iNOS, which was undetectable in unstimulated VSMCs. Exogenous adenosine (1 mM) did not change iNOS level in control VSMCs, but potentiated cytokine-induced iNOS production in diabetic VSMCs. This response was unaffected by the adenosine transport blocker S-(4-nitrobenzyl)-6-thioinosine (NBTI, 10 μM) but was further increased (+45%) by the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1 μM). Similarly, the adenosine precursor AMP (1 mM) enhanced iNOS induction in diabetic but not in control VSMCs. Inhibition of ecto-5'-nucleotidase/CD73 by α,β-methylene-ADP (AOPCP, 200 μM) did not change significantly iNOS protein levels in control and diabetic VSMCs. The adenosine metabolite, inosine (1 mM), was also ineffective in both groups. In the absence of exogenous adenosine, iNOS expression was reduced after treatment with EHNA in control but not in diabetic VSMCs. At the end of 24 h-incubation exogenous adenosine was undetectable in the culture medium of control as well as diabetic VSMCs being converted into inosine and hypoxantine. Treatment with NBTI or EHNA allowed 44% and 26% recovery of added adenosine, respectively, in control VSMCs and 32% and 21% recovery, respectively, in diabetic cells. Similarly, after incubation with AMP, the nucleotide was not detectable and was converted mainly into inosine and hypoxanthine. Treatment with AOPCP allowed 47% recovery of AMP in control, but only 5% recovery in the medium of diabetic VSMCs. When cells from control and diabetic rats were incubated with exogenous inosine, the residual amount of this nucleoside as well as the accumulation of hypoxantine in the medium were quantitatively comparable to those measured after exposing VSMCs to an equimolar concentration of adenosine. These results show that, upon cytokine stimulation, diabetic VSMCs were sensitive to the potential proinflammatory effect of high adenosine concentrations and that alterations in adenosine-related inflammatory pathways may be present in diabetic vascular dysfunction.

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A novel mechanism of estrogen vasoprotection by potentiation of laminar shear stress induction of endothelial heme oxygenase-1

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Premenopausal women are less susceptible to myocardial infarction and stroke than are males of the same age group, an advantage that is lost after menopause (Mendelsohn 2002). In fact, it is recognized that estrogen confers atheroprotection, but the mechanisms are largely unknown. Recently, it was found that estrogen acts on estrogen receptor subtype alpha to up-regulate heme oxygenase-1 (HO-1), a phase II antioxidant enzyme, through prostacyclin signalling in mouse aortic smooth muscle cells (Egan et al. 2004). It has been recently shown that CYP1B1 (member of the cytochrome P450 protein family), involved in estrogen metabolism, is up-regulated by steady laminar shear stress (LSS) (Conway et al. 2009). The aims of the present study were: i) to address whether estrogens may potentiate LSS-induced HO-1 through prostacyclin receptor (IP) activation; ii) to assess the possible contribution of CYP1B1 induction. Thus, in human umbilical vein endothelial cells (HUVECs) exposed to physiologic LSS (10 dyn/cm^2 for 6 h) and in static condition, I assessed: i) the expression of estrogen receptor types alpha and beta; ii) the effect of 17β-estradiol (E_2, 40 nM) on cyclooxygenase-2 (COX-2) expression and on prostanoid generation [6-keto-prostaglandin (PG)$F_{1\alpha}$, the hydrolysis product of prostacyclin; PGE_2 and $PGF_{2\alpha}$]; iii) the effect of E_2 on HO-1 expression; iv) the contribution of prostacyclin signalling using a specific IP antagonist R-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-yl methoxycarbonylamino]-propionic acid (RO3244794, 10 μM) (Bley et al. 2006). HUVECs (at passage level 2 or 3) were grown in DMEM-medium 199 (50% v/v), supplemented with endothelial cell growth supplement (50 μg/ml), heparin (100 U/ml), pen-strep 100 U/ml, glutamine 1%, and fetal calf serum (FCS) 15%. Confluent monolayers (0.8 to 1x10^6 cells per plate) were placed in a parallel plate perfusion chamber and perfused at a shear rate of 1400 sec^{-1}. HUVEC monolayer was exposed to a shear stress of 10 dyn/cm^2 in 7 ml DMEM-medium 199, supplemented with pen-strep 100 U/ml, glutamine 1%, and FCS 5%. The entire apparatus was maintained in a humidified 5% CO_2/95% air atmosphere at 37°C for 6 h. The perfused medium was assayed for 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ by radioimmunoassay and cell lysates were analyzed for COX-2 and HO-1. In parallel, HUVECs were cultured in static condition for 6 h. Western blot analysis showed that HUVECs expressed ER-alpha and ER-beta receptors. In HUVECs cultured in static condition or under LSS after the treatment with 40 nM E_2 for 6 h, COX-2 was not modulated. This was associated with no significant change of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ biosynthesis. In HUVECs under LSS, HO-1 was up-regulated and this effect was significantly ($P<0.05$) further enhanced by E_2 (ratio of HO-1/β-actin optical density 1.70±0.88 in HUVECs under LSS vs 3.90±0.55 in the presence of E_2). To verify the involvement of prostacyclin on the induction of HO-1 by E_2, I used a specific IP antagonist, RO3244794. RO3244794 caused a significant reduction of HO-1. However, the block of IP did not prevent E_2-dependent induction of HO-1 in HUVECs exposed to LSS. Finally, CYP1B1 was induced in HUVECs under LSS and this effect was countered by E_2. In summary, E_2 potentiated HO-1 induction in HUVECs exposed to steady LSS through a mechanism that did not involve prostacyclin generation and activity. Interestingly, E_2 interfered with the induction of CYP1B1 which contibutes to E_2 metabolism producing 4-hydroxyestradiol (Hayes et al. 1996). In conclusion, I have enlightened a novel protective role of E_2 involving the overexpression of the cytoprotective HO-1. The possible contribution of reduced E_2 metabolism requires further investigation.

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Effects of lithium and carbamazepine on experimental model of mental disorder

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Lithium is regarded as the gold standard medicament in the treatment of bipolar disorder and it reduces the risk of attempted or completed suicide, while other mood stabilizers, such as anticonvulsants, lack substantial evidence of antisuicidal activity. Anhedonia in humans is an established suicidal risk factor in depressed, bipolar, and schizophrenic subjects, and to our knowledge no studies have compared the possible antianhedonic activity of mood stabilizers. The concept of anhedonia implies not only the inability to perceive a pleasurable stimulus, but also a reduced capacity to acquire and/or emit behaviours aimed at approaching or earning a reward, and it has been often associated with a deficit in cortical mesolimbic dopaminergic transmission (Scheggi et al. 2010). Dopamine does not directly interfere with the hedonic response to a rewarding stimulus; however, the phasic increase in mesolimbic dopamine output in response to a relevant stimulus is dependent on and is an indicator of its actual incentive value. As a corollary, it has been proposed that animals like the effects of dopamine signalling and that they will engage in activities directed towards restoration of dopamine transmission. A deficit in this spontaneous tendency may result in anhedonia.
Exposure to palatable food is widely used in experimental protocols since it elicits consistent behavioural and neurochemical responses. We only use non food-deprived rats since in these animals the emotional value of food has a prevalent hedonic component. Rats are very fond of vanilla sugar (VS) and consumption of 4-5 small VS pellets increases dopaminergic transmission in discrete frontal mesolimbic areas, such as the medial prefrontal cortex (mPFC) and the shell portion of the nucleus accumbens (NAcS). Only rats that show a dopaminergic response in the mPFC and NAcS after a VS meal consistently acquire an instrumental behaviour based on the reinforcing properties of VS pellets, the earning of which is made contingent on the choice of one of the two divergent arms of a Y-maze (VS sustained appetitive behaviour, VAB). VAB acquisition models a behaviour capable to activate dopaminergic transmission in the frontal mesolimbic areas (Gambarana et al. 2003).
Long-term carbamazepine (6 mg/kg b.i.d.) or lithium (8 mEq/kg b.i.d.) administration in rats induced hyporeactivity to aversive stimuli and a tonic decrease in dopamine output in NAcS (Masi et al. 2000). However, long-term lithium or carbamazepine administration produced divergent behavioural and neurochemical responses when rats were exposed to a palatable food. Thus, while carbamazepine-treated rats showed reduced dopaminergic response to VS consumption and did not acquire VAB, lithium-treated rats showed intense dopaminergic response to VS consumption and acquired VAB as efficiently as control rats. Moreover, after VAB acquisition, lithium-treated rats showed basal dopamine output in the NAcS and ability to avoid aversive stimuli similar to those of control rats. These results raised the issue of whether the observed differences were dependent on an anhedonic effect of carbamazepine, or whether an antianhedonic activity of lithium could be hypothesized. In order to clarify this issue, I studied the effects of repeated lithium administration in cocaine-sensitized rats that configure a model of anhedonia. Cocaine-sensitized rats show no dopaminergic response to a VS meal and do not acquire VAB. Daily lithium administration (8 mEq/kg b.i.d.) 10 days before and during VAB training completely reinstated the competence to acquire VAB in cocaine-sensitized rats. Thus, the present results suggest that lithium is endowed with anti-anhedonic activity.

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Evaluation of the neuroprotective effect of parkin against α-synuclein-induced neurotoxicity in a rat model of Parkinson's disease

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Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder characterized by a profound and selective loss of nigrostriatal dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), leading to a loss of dopamine in the target structure striatum and development of motor symptoms such as bradykinesia, rigidity and tremor (Kim et al. 2009). The etiology of PD is still largely unknown. At least six different casual genes linked to rare familial forms of PD have been identified, while late-onset idiopathic PD is thought to result from the interplay between predisposing genes and environmental factors. In particular, two missense mutations (A30P and A53T) in the *α-synuclein* (α-syn) gene have received great attention with the discovery that abnormal metabolism and accumulation of α-syn in dopaminergic neurons leads to both sporadic and familial forms of PD (Batelli et al. 2008; Recchia et al. 2008). Parkin functions as an E3 ubiquitin ligase: loss of its activity seems to cause an autosomal recessive form of PD (Von Coelln et al. 2004). We have recently described a hemi-parkinsonian rat model, based on the stereotaxic injection of TAT-α-syn-A30P in the SNpc of the right hemisphere (Recchia et al. 2008). The TAT sequence allows diffusion of the fusion protein across the neuronal plasma membrane and results in a localized dopaminergic loss (Recchia et al. 2008).
The research project is designed to examine possible neuroprotective effects of TAT-parkin against α-syn-induced neurotoxicity in this model. Rats were stereotaxically injected with TAT-α-syn-A30P, TAT-parkin, or both. At different times after injection, all animals were subjected to behavioural testing to evaluate impairment in motor function. Then, dopaminergic cell loss was evaluated with the apomorphine-induced rotation test and tyrosine hydroxylase immunohistochemistry. In order to better visualize the extent of the lesion, all animals were sacrificed and the brain processed for immunohistochemical analysis with a specific primary antibody against TH. The α-syn-parkin-based model better reproduces the pathophysiology of PD and could be of utility to understand the mechanisms that lead to dopaminergic neurodegeneration. Moreover, it could help identify disease-modifying strategies as opposed to therapies which provide only symptomatic relief.

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Characterization of interleukin-18 system in the mouse central nervous system: a potential novel pharmacological target for the therapy of multiple brain pathologies?

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Cytokines are pleiotropic proteins originally characterized as immune modulators coordinating the host response to infection but subsequently have been found to be involved in the normal ongoing signalling between cells of non-immune tissues including the nervous system. Beside the better known detrimental action during brain disease, emerging evidence has referred that cytokines cover an important role in neural development as well as in the maintenance of the healthy brain during aging. Moreover, a dysregulation of the fine balance between pro-inflammatory and anti-inflammatory cytokines depending on the context, stimuli, and timing can contribute to distinct neurodegenerative and neuropsychiatric diseases often accompanied by neuroinflammatory processes (Farooqui et al. 2007). Among pro-inflammatory cytokines expressed constitutively in the brain, interleukin (IL)-18 has been proposed as important mediator of the communication between the immune and the central nervous system (CNS). Clinical and pre-clinical data demonstrated an involvement of IL-18 in the etiology of a number of pathological conditions affecting the CNS including Parkinson and Alzheimer's diseases, psychiatric disorders and multiple sclerosis. Moreover, experimental evidence indicates that IL-18 possesses biological functions in the healthy and diseased brain such as induction of sleep, attenuation of the long-term potentiation in the hippocampus, suppression of appetite, regulation of hypothalamic-pituitary-adrenal axis activity, anxiety, and spatial learning (Alboni et al. 2010). Nevertheless, the IL-18 system has been poorly investigated in the brain and the mechanisms through which IL-18 may regulate CNS functions remain largely unknown. Peripherally, IL-18 action is mediated through binding to a specific receptor (IL-18R) complex, a heterodimer composed of an α subunit (IL-18Rα) that binds IL-18, and a β subunit (IL-18Rβ) which initiates signal transduction. We have recently demonstrated that IL-18Rα and its putative decoy isoform IL-18Rα type II are expressed throughout the mouse CNS, mainly with neuronal localization, suggesting that IL-18 may directly modulate neuronal function (Alboni et al. 2009). Considering the co-expression of IL-18Rβ fundamental to generate a functional receptor, the first aim of this work was to investigate in the mouse brain the expression of the canonical IL-18Rβ as well as its short variant already described in rat and human (Andre et al. 2003). Using a multi-methodological approach, we demonstrated that: i) a small IL-18Rβ variant is expressed in the mouse brain although at lower basal levels than the full length IL-18Rβ; ii) the canonical IL-18Rβ is expressed in the CNS particularly in areas and nuclei belonging to the limbic system as previously observed for IL-18Rα. The expression of a functional IL-18R in these limbic regions suggests that IL-18 may act directly regulating the brain functions supported by this system. As second step, we meant to investigate in the mouse brain the effects on the IL-18 system of a peripheral administration of lipopolysaccharide (LPS), the bacterial endotoxin known as strong inducer of pro-inflammatory cytokines. Notably, LPS is able to trigger also the "sickness behaviour" syndrome, a condition mediated by pro-inflammatory cytokines characterized by behavioural and physiological symptoms commonly associated to infectious diseases. We found that 3 h after LPS injection (100 μg/mouse), a marked increase of full length and small IL-18Rβ expression occurred in specific brain areas whereas the putative decoy receptor IL-18Rα type II mRNA levels were increased in the cerebral cortex and in hippocampus. In summary, these findings may indicate that IL-18 system possesses a high grade of complexity and a finer regulation than previously believed and that a systemic stimulus such as a LPS challenge promotes a regulation of IL-18 system in the mouse brain. Hence, the IL-18 system components may represent a novel target for new and more effective drugs acting in those neurodegenerative and neuropsychiatric diseases for which it has been described an involvement for IL-18.

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Anti-Ro/SSA antibodies and arrhythmogenesis in the adult heart

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My doctoral work has dealt with the arrhythmogenic role of anti-Ro/SSA antibodies in adult patients with autoimmune diseases. Anti-Ro/SSA antibodies represent the result of the autoimmune response against the so-called Ro antigen, an intracellular ribonucleoprotein constituted by two subunits of 52 and 60 kDa of molecular weight, respectively, and a molecule of small cytoplasmic ribonucleic acid (Franceschini et al. 2005). A large body of evidence links the trans-placental passage of maternal anti-Ro/SSA antibodies with the risk for the newborn to develop a rare but severe syndrome, named neonatal *lupus*, in which the main challenge is the congenital heart block.

Differently from foetuses and newborns, the adults seem to show a peculiar resistance to the arrhythmogenic effects of anti-Ro/SSA antibodies. Indeed, several recent studies also performed in our Institution strongly suggest the existence of a clinical and pathogenetic association between some rhythm disturbances and anti-Ro/SSA antibodies in adult subjects, thereby unexpectedly reopening the debate on this topic (Lazzerini et al. 2009). In particular, the prolongation of the QTc interval appears the most frequent abnormality (20-50% of the cases) observed in adults with circulating anti-Ro/SSA antibodies, with some data suggesting an association with an increased risk of ventricular arrhythmias, also life threatening.

On these bases, we suggested the possibility of a selective blocking activity of the anti-Ro/SSA antibodies on the rapid component of the delayed rectifier potassium current (I_{Kr}), which in turn represents the main target of the drug-induced acquired long QT syndrome (Lazzerini et al. 2009). Recent evidence firmly supports such a hypothesis. In fact, Nakamura et al. (2007) demonstrated a selective inhibitory effect on hERG potassium channel (conducting the I_{Kr} current) by serum or IgG from an anti-Ro/SSA positive young female with extreme QTc prolongation (700 msec).

On this basis, indicating a specific and direct interaction autoantibody-ion channel, we hypothesize that both (i) the anti-Ro/SSA circulating levels, and consequently the number of potassium channels blocked on the cardiomyocyte membrane, and (ii) the specific anti-Ro/SSA antibody subtype involved (i.e. recognizing the 52 or 60 kDa subunit) may represent a crucial factor to produce (or not) a significant interference on cardiac cell electrophysiology expressed in terms of QTc prolongation. This consideration, on which currently are focused our research, might explain why only about 20-50% of the anti-Ro/SSA positive patients presented a QTc prolongation. To confirm this hypothesis we studied 49 patients (7 ♂; mean age 42.6±12.4 years) affected by several types of CTDs. All the patients underwent resting electrocardiogram to measure the QTc interval, and measurement of circulating level of anti-Ro/SSA antibodies (total, anti-60 kDa, and anti-52 kDa) by EIA method. Our results indicate a direct significant correlation between QTc duration and circulating antibody concentration with the total anti-Ro/SSA (r=0.35, P=0.013), and more significantly with anti-Ro 52 kDa level (r=0.38, P=0.007). On the contrary, no association was demonstrated with anti-Ro 60 kDa serum concentration.

These data, possibly suggesting a specific molecular mimicry between the Ro 52 protein and the hERG channel, may help explain why only a percentage of patients displaying anti-Ro/SSA positivity develop a prolonged QTc. Further experiments of Western blotting are now in progress to evaluate if actually purified IgG from anti-Ro/SSA positive patients displaying high level of anti-Ro 52 kDa antibodies and QTc prolongation are able to specifically recognize hERG potassium channel.

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The flavonoid quercetin stimulates vascular smooth muscle $Ca_v1.2$ current: new insight into its mechanism of action

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Quercetin is a natural polyphenolic flavonoid widely found in edible plants (i.e. fruits, vegetables, herbs, grains) and beverages (i.e. tea, red wine). Interest in dietary polyphenols has greatly increased owing to their possible beneficial implications in human health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies (Scalbert et al. 2005). Recent findings from this laboratory have highlighted vascular ion channels as important pharmacological targets for flavonoids. Specifically, while (±)-naringenin was able to stimulate $K_{Ca1.1}$ channel current ($I_{K1.1}$; Saponara et al. 2006), and quercetin and myricetin were shown to stimulate $Ca_v1.2$ channel current ($I_{Ca1.2}$) (Saponara et al. 2002; Fusi et al. 2005), cardamonin behaved like a bifunctional vasodilator capable of inhibiting $I_{Ca1.2}$ and stimulating $I_{K1.1}$ current (Fusi et al. 2010). Furthermore, the hypothesis that flavonoids can affect vascular tone and cardiac function by direct targeting cardiovascular ion channels has been strengthened by a recent review of Scholz et al. (2010).
Radioligand binding experiment is the first choice technique for providing a direct evidence of the existence of a receptor/binding site. However, it is well know that drug interaction with and drug binding to $Ca_v1.2$ channel depend on the experimental conditions that determine its state (Zahradnikova et al. 2007). Therefore, $I_{Ca1.2}$ recording from operational $Ca_v1.2$ channels (as is the case with the patch-clamp technique) may represent a more physiological approach allowing an in-depth analysis of flavonoids target site. Following this theory, Saponara et al. (2008) demonstrated that nutritionally meaningful concentrations of quercetin limited the responsiveness of vascular $Ca_v1.2$ channel to the pharmacological stimulation operated by the dihydropyridine Bay K 8644. Based on this experimental approach, the aim of my first year doctoral work was to characterize the electrophysiological effects of quercetin on vascular $I_{Ca1.2}$. $I_{Ca1.2}$ was recorded using the conventional whole-cell patch-clamp method in single smooth muscle cells isolated from the rat tail main artery. Under control conditions, $I_{Ca1.2}$ elicited with 250 ms clamp pulses (0.067 Hz) to 10 mV from a V_h of -50 mV achieved a stable intensity about 10 min after the whole-cell configuration had been obtained. Neither $I_{Ca1.2}$ intensity nor the current-voltage relationship changed over the next 40 min of recording. When cells were challenged with 0.02% (v/v) dimethyl sulfoxide, the vehicle used to dissolve quercetin, $I_{Ca1.2}$ decreased by 23% after 30 min (n=8, $P<0.05$). In cells treated with 10 µM quercetin, a gradual increase of the current intensity was observed; this gained a maximum value in about 4 min, remained stable for about 10-12 min, and then slowly decreased back to control value. This phenomenon can be ascribed to several different mechanisms: a) quercetin possibly reacts with additional targets, beyond the $Ca_v1.2$ channel, and this causes a late current inhibition; b) the large Ca^{2+} influx occurring during repetitive stimulation in the presence of quercetin leads to an accumulation of Ca^{2+}-dependent inactivation of the current; c) others. Further experiments performed in the presence of Ba^{2+} as the charge carrier are in progress in order to clarify this point.
In conclusion, understanding the mechanism(s) responsible for quercetin-induced stimulation of $I_{Ca1.2}$ is of great interest in view of a recent finding indicating that activation of $Ca_v1.2$ channels increases the expression of a group of genes required for neuronal survival after ischemia-reperfusion injury (Li et al. 2007).

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Pharmacologically active microcarriers as scaffolds for endothelial progenitor cells

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Endothelial progenitor cells (EPCs) can contribute to neovascularisation by their physical incorporation into the vessels during their formation, or by a paracrine release of growth factors. The multiple issues related to inoculate EPC has led to the establishment of support and vehicle for autologous stem cells. These include microparticles polymer (PAM) of 60 μm diameter, biodegradable and biocompatible, coated with fibronectin and poly-D-lysine sequences (Tatard et al. 2005).
The aims of this research were: i) to assess the adhesion of human EPCs to PAM; ii) to assess the functional properties of PAM functionalized with vascular endothelial growth factor (VEGF).
EPCs were obtained from *in vitro* differentiation of human peripheral mononuclear cells (PBMC) of healthy donors in endothelial growth medium supplemented with angiogenic factors (Bellik et al. 2008). The pre-endothelial phenotype was characterized by flow cytometry analysis of antigen expression (VEGFR-2, VE-cadherin, vWF) and by immunocytochemistry. It was firstly optimized a protocol to assess EPC adhesion to PAM. The optimal EPC concentration found was 125,000 cells/0.5 mg PAM. Then, time-course (6 h-4 days) experiments were performed in order to characterize EPC adhesion. EPCs were able to adhere to PAM within few hours. This adhesion was increased if PAM were further coated with fibronectin. Moreover, the adhesion to PAM was also correlated to the EPC phenotype. Pharmacologically active PAM, which release VEGF, increased the ability of EPC to adhere to them. Moreover, they significantly stimulated the migration of mature endothelium (HUVEC) without any effects on EPCs. There results might add new insight into the role of scaffolds cell-mediated regenerative medicine.

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Inhibition of P-glycoprotein by novel MDR reverting agents for cancer therapy

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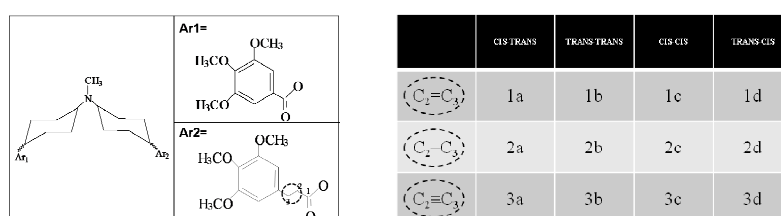
Many tumor cells become resistant to commonly used cytotoxic drugs due to the overexpression of ATP-binding cassette (ABC) transporters. P-glycoprotein (Pgp; MDR1, ABCB1) and MRP1 (ABCC1) have been demonstrated to pump a wide selection of the most commonly used cancer drugs (Fusi et al. 2006). Several generations of inhibitors of Pgp have been examined in preclinical and clinical studies. The main problems associated with the development of these drugs seem due to poor specificity, low potency, and interference with physiological functions (O'Connor 2007). Four geometrical isomers characterized by N,N-bis(cyclohexanol)amine scaffold non-symmetrically esterified with two different aryl acids (Ar_1=3,4,5-trimethoxybenzoyloxy; Ar_2=3-(3,4,5-trimethoxyphenyl)acryloyloxy) (**1a-d**) (Figure 1) (Martelli et al. 2009) were investigated as Pgp inhibitors by measuring cytofluorimetrically the retention of rhodamine 123 (R123) in human MDR1-gene transfected mouse T-lymphoma L5178 cells (Saponara et al. 2006). **1a** and **1d** inhibited Pgp with IC_{50} values of about 1 nM , while **1b** IC_{50} value was 100 nM. Since upon cell washing reversion of **1d** Pgp inhibition was incomplete, suggesting an irreversible binding with the target, the role of double bond present in Ar_2 was investigated inserting as Ar_2 a 3-(3,4,5 trimethoxyphenyl)propionyloxy or a 3-(3,4,5-trimethoxyphenyl)propynoyloxy moiety. Two sets of four isomers were thus obtained, where the double bond of 1a-d was substituted by a single (**2a-d**) and a triple bond (**3a-d**), respectively. **2a-d** were nearly equipotent, **2d** and **2c** showing IC_{50} values two orders of magnitude greater than the double-bond counterparts. On the contrary, **3a-d** were very effective, the most active **3d** and **3c** showing a potency greater than that of double-bond counterparts (**1d** and **1c**). Concentration-inhibition curves of **1c** and **3d** exhibited a biphasic behaviour suggesting the existence of two binding sites for them in the recognition domain of Pgp. The persistence of inhibition of Pgp-mediated R123 cell efflux by **1c**, **1d**, **3c**, and **3d** resulted to be intermediate between that caused by cyclosporin A and GF120918. Finally, the competition between R123 and some derivatives for the Pgp binding site was also studied. L5178 MDR1 cells were incubated with increasing concentrations of **1c**, **1d**, **2c**, **2d**, **3c**, and **3d** in presence of 5 or 50 μM R123. Results showed that the highest R123 concentration caused a parallel leftward shift (**1c**, **1d**, **2c**, **2d**, and **3c**) or a rightward shift (**3d**) of the concentration-response curve with no depression of the maximum, indicating a positive cooperativity between R123 and some of these Pgp inhibitors.
In conclusion, **1c** and **3d** possess a remarkable potency as inhibitors of Pgp function as they showed a half-maximum activity in the low nanomolar range, i.e. much lower than that of the most active compounds studied until now. **1c** and **1d** appear promising leads for the design of effective, novel MDR reverters in cancer cells.



	CIS-TRANS	TRANS-TRANS	CIS-CIS	TRANS-CIS
$C_2=C_3$	1a	1b	1c	1d
C_2-C_3	2a	2b	2c	2d
$C_2\equiv C_3$	3a	3b	3c	3d

Figure 1: structures of the compounds investigated.

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In vitro characterization of the heterogeneity of third trimester human amniotic fluid cells and their use in a mouse model of neurodegenerative disease

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Since its devastating consequences, spinal cord injury (SCI) has polarized the efforts of many research groups all around the world. Pharmacological approaches aimed at preventing secondary degeneration in SCI have been recently flanked by cellular methods and stem cells have been studied as a new tool for SCI therapy. Latest evidences indicate that stem cells could be a good tool to reduce secondary degeneration in SCI.
Multiple cell types derived from the developing fetus compose the cellular compartment of the amniotic fluid. For instance, epithelioid cells derive from fetal skin and urinary tract, amniotic fluid specific cells come from fetal membranes, and trophoblast and fibroblastic cells derivate from fibrous connective tissue and dermal fibroblasts. These cells are able to differentiate in adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic lineages, indicating their multipotency.
The main goal of this study was the characterization of the cells from the third trimester amniotic fluid obtained from programmed caesarean births (instead of cells usually retrieved from amniocentesis) and tests their therapeutic potential in a mouse model of SCI.
Different populations of adherent cells were isolated from eleven human amniotic fluids and they were characterized for *in vitro* proliferation and differentiation potential. The antigenic profile was performed either by immunocitochemistry and citofluorimetric analysis. In particular, four cultures were deeply investigated and, by immunoistochemical analysis, all of them showed the expression of neural markers such as nestin, β tubulin III, and GFAP. After citofluorimetric analysis, the samples showed a noticeable expression of adult mesenchymal markers (CD146+, CD73+, CD105+, CD90+) directed to the muscle-neural lineage (CD146+, NG2+, CD56+) (#3.5, #3.6, and #9.1); one of them also expressed CD117 (#3.6); culture #1.1, instead, showed a mesenchymal phenotype directed to the perivascular lineage (CD146+, CD90+, CD73+). From a morphological point of view, we were able to identify a new sub population of small cell spindle-like shaped which were highly represented in #9.1 culture.
We decided to use four populations (#3.6, #3.5, #1.1, and #9.1) to transplant spinal cord injured mice. One week after transplantation immunosuppressed animals were intravenously injected with cells or PBS (controls) and motor recovery of the transplanted animals was studied for other 28 days by open field analysis.
The animals transplanted with culture #3.5 and #3.6 showed a significant motor recovery, higher than animals treated with PBS only; animals transplanted with cultures #1.1 and #9.1, instead, did not show any significant enhanced performance as compared to PBS treated animals.
We tried then to investigate the reasons of these different results and, after histological analysis, we noticed that cultures #3.6 and #3.5 (the "therapeutic" lines) induced a better preservation of the myelin in the ventral white matter within the lesion site than PBS animals. Moreover, in these animals we could appreciate an increased angiogenesis (by lectin staining) in the peri-injured area, one month after lesion, as compared to controls. It was also possible to find transplanted cells at the lesion site and in a region of 4 mm rostrally to the injured area.
An additional intriguing element of the story is that the therapeutic lines all showed the highest expression levels of the common marker NG2 that seems to play a crucial role in the developing and mature central nervous system but also in the process of angiogenesis. For this reason, we are now investigating the expression levels of HIF and VEGF at the site of injury by real time PCR and we are now planning to depict the hypoxia cascade modifications in our model to understand if transplanted cells could, by this pathway, play a role in the myelin preservation. This could be the physiological phenomenon responsible of the behavioural improvements observed.

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Antinociceptive effects of intrathecally administered nociceptin/orphanin FQ receptor agonists in the rat: tail flick test

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Nociceptin/orphanin FQ (N/OFQ) regulates various biological functions at central and peripheral levels, including pain, anxiety, stress, learning, memory, food intake, and drug addiction, by selectively activating a G-protein coupled receptor named N/OFQ peptide (NOP) receptor (Lambert 2008). As far as pain transmission is concerned, the role of the N/OFQ-NOP receptor system is complex. The majority of the available results indicate that N/OFQ signalling has opposite actions at supraspinal and spinal levels being pronociceptive in the brain and antinociceptive in the spinal cord (Zeilhofer et al. 2003). Recently, several chemical modifications aimed at increasing potency and resistance to degradation have been combined into the N/OFQ sequence leading to the discovery of a novel series of compounds, in particular the full agonist UFP-112 and the partial agonist UFP-113 (Arduin et al. 2007). The aim of this study was the investigation of the effects of N/OFQ (1, 10, and 100 nmol), UFP-112 (1, 3, 5, and 10 nmol), UFP-113 (10, 30, 50, and 100 nmol) and morphine (0.1, 1, 3, and 10 nmol) given intrathecally (i.t.) in the rat tail flick test.
For intrathecal catheterization, male Wistar rats (150-170 g) were anaesthetized by ketamine plus xylazine. The lumbar puncture catheter was acutely implanted using a slight modification of a method by Størkson et al. (1996). By raising the ventral iliac spines, where a longitudinal incision was made, the lumbar part of the spinal column was rendered temporarily kyphotic (by locating a small cushion under the rat's belly), and the needle could be advanced 2-4 mm in the narrow space between L5 and L6. The correct intrathecal localization was confirmed by a tail flick or a paw retraction by easy insertion of the catheter through the cannula. The catheter was made of a polyethylene tube, outer diameter 0.6 mm (PE-10) and was cut to 14 cm, including 4 cm of the subarachnoid space-placed part. After a four-day recover, drug or saline was i.t. injected in a volume of 10 µl, followed by 10 µl of sterile saline to flush the catheter. Rats were submitted to the tail flick test as described by Tian et al. (1997); tail flick latencies were measured at t=0 (pre-drug latency), and 15, 30, 60, and 120 min after treatment. The drug effects were calculated as % maximal possible effect (MPE) (control latencies=3-5 s, cut-off time=20 s) and MPE data used for calculating the area under the curve (AUC) over the 15-120 min period of testing. At the end of the experiment, lidocaine (15 µl from a 20 mg/ml solution) was given to verify the correct placement of the catheter.
Results indicate that morphine, as a reference analgesic, is able to induce a significant antinociceptive effect from the dose of 3 nmol [(AUC 2628±417 vs 419±62 (controls), P<0.01]. The effect of morphine was stable over time and still evident at the end of the experiment. N/OFQ produces a dose-dependent antinociceptive effect showing significant effects at the highest dose used (AUC, N/OFQ 100 nmol: 3689±529 vs controls P<0.01). The antinociceptive effect of the peptide peaked at 30 min and declined over the time course of the experiment. At the dose of 5 nmol, the high potent NOP agonist UFP-112 showed an antinociceptive action comparable to that of morphine 10 nmol (AUC 5827±846, P<0.01 vs controls) both in terms of amount and time course of effect. However, at the highest dose, i.e. 10 nmol, UFP-112 caused motor dysfunction. UFP-113 elicited clear antinocicetive effects at the dose of 50 nmol (AUC 7404±1014, P<0.01 vs controls) which were stable over time; similarly to UFP-112, the highest dose of this peptide provoked a slight paralysis of the animal hind limbs. Thus UFP-112 and UFP-113 mimicked the antinociceptive action of N/OFQ with higher potency and displayed, similar to morphine, long lasting effects. The results are instrumental for selecting the adequate doses of drugs to be used in chronic studies aimed at evaluating the development of tolerance to the antinociceptive effects of these peptides. In conclusion, the present experiments confirmed that the selective activation of spinal NOP receptor elicits a clear antinociceptive action in rodents. These findings together with those coming from non human primates studies (Ko et al. 2006; 2009) suggest that NOP selective agonists as worthy of development as innovative spinal analgesics.

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Vascular smooth muscle cell and dendritic cell interactions in pathological vascular remodelling

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Vascular remodelling is a chronic multi-factorial disease in which systemic or local inflammatory pathogenetic mechanisms play a key role (Libby 2006). Atherosclerotic lesion development and its complication are characterized by inflammation and immunological mechanisms. It has been become evident that the cellular components of both innate and adaptative immunity, such as dendritic cells (DCs), are also involved in driving the chronic vascular inflammation which characterizes this disease (Bobryshev 2005). DCs number increases during atherosclerosis and is abundant in the shoulder regions of plaques in which they co-localize with activated T cells. However, the pathogenetic and immunomodulant role of vascular DCs in the activation of vascular smooth muscle cells (VSMCs) is still unclear. Since no information are still available on the role of DCs on the activation of these cells, the aim of the study was to assess the cellular and molecular mechanisms responsible for the interaction between DCs and VSMCs.
DC were generated from peripheral blood of healthy donors. Monocytes were sorted by $CD14^+$ magnetic beads selection, cultured for 6 days in medium supplemented with GM-CSF and IL-4, followed by a further maturation stimuli (IL-6, IL1β and TNF-α). Mature DCs expressed high levels of co-stimulatory molecules CD80, CD86, the maturation marker CD83, and HLA-DR. The stimulation of human coronary smooth muscle cells (CASMCs) with inflammatory cytokines increased the expression of CD40 and stimulated the appearance of CD40L. Mature DCs were able to adhere to CASMCs while immature DCs did not. Pretreatment of CASMCs with inflammatory cytokines increased DC adhesion to CASMCs. ICAM-1, VCAM-1, and the subunit integrins CD11c and CD18 were involved in this process, while DC-SIGN did not. These findings suggest that the inflammatory environment may stimulate the interaction between DCs and VSMCs thus driving vascular remodelling.

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12-13K-RAS kit: an IVD for the detection of mutations in the *K-ras* oncogene by amplification of DNA in the codon 12 and 13 region and RFLP

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It is known that multiple genetic alterations can contribute to the development of most cancers. These mutations are mostly somatic; mutations of the germinal line are rare and involve less that 1% of all human cancers. Among the gene-related cancers, the *ras* family ones are very well characterised.
Harvey-ras (*H-ras*), *Kirsten-ras* (*K-ras*), and *N-ras* (found in active form in neuroblastoma) are the three functional genes of this family. They code for 21 KDa proteins that have important roles in signal transduction and cell proliferation; they are located in the inner side of the cell membrane and ubiquitously expressed. They belong to the G-protein family as they link with high affinity and specificity GTP and GDP and show GTPase activity: they switch from the GDP-linked inactive state to the GTP-linked active state.
It was demonstrated in several kinds of human cancers that the RAS proteins (p21RAS) can acquire transforming power as a consequence of point-mutations in their respective genes (Kiaris et al. 1995). These mutations decrease the GTPase activity of the RAS protein and increase the GTP to GDP reaction rate, so the active form of p21RAS, which is the form that stimulates growth and cell differentiation (Satoh et al. 1992).
Among the *ras* genes family, the mutated *K-ras* gene is found in the earliest stages of the development of some cancers; therefore, these mutations can be biomarkers for early diagnosis and prevention of those cancer diseases (Ronai 1992). *K-ras* gene mutations are common and well characterised in pancreas, colon, and lung carcinomas, that are responsible for the greatest number of cancer deaths worldwide (Broder 1993; Parker et al. 1996). Three hot spots in the *K-ras* gene at codon 12, 13, and 61 have been characterised. The frequency of mutations, at the level of the 3 codons, are the following in percentages: 70-75% in codon 12; 20-25% in codon 13; <5 % in codon 61. The highest incidence has been found in pancreas adenocarcinoma, in which the specific mutation is detectable in about 90% of the analysed samples. The same mutation was described in 40% of colon adenocarcinomas, in 30% of lung, and 50% of follicular thyroid adenocarcinomas.
During the American Society of Clinical Oncology in 2008, evidence was presented in which patients with colon tumours in advanced stages would not receive any benefit from the administration of cetuximab (Erbitux) and panitumumab (Vectibix), if there were mutations in the *K-ras* gene. The k-ras mutational state is thus a predictive marker for the anti-tumoral therapy. Several methods based on gene amplification have been used for the detection of mutations in *K-ras* gene (Minamoto et al. 2000). Since the mutations at codon 12 cause a specific restriction site deletion, they can be easily demonstrated at the molecular level by amplification of the region of interest followed by digestion with a specific restriction enzyme and RFLP analysis. The 12-13K-RAS kit is an IVD for the detection of mutations in the *K-ras* oncogene by amplification of DNA in the codon 12 and 13 region and RFLP. The molecular strategy adopted in this kit for detection of mutations at the 12 and 13 codons of *K-ras* gene consists in amplification of previously extracted DNA with specific primers flanking the region of interest, followed by an enzymatic digestion (RFLP) for typing the mutation. The mutation at codon 12 and 13 causes a restriction site deletion that can be easily demonstrated at the molecular level by digestion with a specific restriction enzyme followed by high resolution agarose gel electrophoresis.The mutation can be identified by restriction fragments size analysis (electrophoretic pattern).

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A novel mechanism of action of indoleamine 2,3-dioxygenase, a crucial mediator of immune suppression

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My doctoral work has dealt with indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the initial and rate-limiting step of tryptophan catabolism in a specific pathway resulting in a series of metabolites collectively known as kynurenines. Although the same reaction is catalyzed by tryptophan 2,3-dioxygenase (TDO), IDO alone has been recognized as an authentic metabolic regulator of immunity in mammalian pregnancy as well as in infection, autoimmunity, chronic inflammation, allergy, transplantation, and neoplasia (Grohmann et al. 2003; Mellor et al. 2004). The suppressive effects of this enzyme have been explained by tryptophan deprivation and the production of regulatory metabolites. By mapping the recently defined human IDO structure, we found that IDO contains two tyrosines within two distinct canonical immunoreceptor tyrosine-based inhibitory motifs or ITIMs (ITIM1, VPY115CEL; ITIM2, LLY253EGV). A prototypic ITIM is the I/V/L/SxYxxL/V sequence (Ravetch et al. 2000), where x denotes any amino acid. These domains, although quite distant in the primary amino acid sequence, are very closed in the three-dimensional structure of the human enzyme and are present not only in human but also in mouse, rat, and dog IDO. The occurrence of ITIM motifs in IDO raised the possibility that the enzyme participates in inhibitory signalling that may add to tryptophan degradation-dependent regulation. The inhibitory function of ITIMs is mediated by recruitment and activation of protein-tyrosine phosphatases SHP-1 and SHP-2, all containing SH2 domains with high affinity for ITIM phosphotyrosine (Billadeau et al. 2002). To verify whether the putative ITIM domains in IDO could represent docking sites for SH2 domain-containing molecules, biotinylated peptides containing phosphorylated or unphosphorylated mouse IDO ITIM1 or ITIM2 sequences were used to pull-down SHP-1 and SHP-2 in both mouse plasmacytoid dendritic cells (pDCs) and P1 tumour cells. To demonstrate any direct interaction between whole IDO protein and SHP-1 or SHP-2, we performed co-immunoprecipitation experiments with P1 cells stably transfected with IDO-Flag (P1.IDO-Flag) and treated with pervanadate (PV; a protein-tyrosine phosphatase inhibitor). We found that low levels of endogenous SHP-1 and SHP-2 - co-immunoprecipitated with IDO - were detectable in the absence of PV treatment. Yet, co-immunoprecipitation efficiency greatly increased in the presence of the phosphatase inhibitor. These data suggested that IDO contains ITIM-containing sequences that represent docking sites for protein tyrosine phosphatases. To evaluate the relative contributions of IDO ITIM1 and ITIM2 to phosphatase binding and activation, we generated a series of constructs encoding Flag-tagged IDO mutants lacking either the ITIM1 tyrosine (IDOY115F), the ITIM2 tyrosine (IDOY253F), or both (IDOY115F/Y253F). In addition, we generated a mutant lacking the histidine required for IDO catalytic activity (IDOH350A). All mutants were singly transfected either stably into P1 cells or transiently into pDCs, and their enzyme activity was evaluated in terms of kynurenine production. We found that Flag-tagged wild-type IDO (wtIDO) or tyrosine IDO mutants determined the production of comparable levels of kynurenine in different transfectants within the same type of cell. In contrast, expression of IDOH350A did not cause detectable production of kynurenine in P1 cells. The immunoprecipitates from transfected P1 cells were assayed for the presence of tyrosine phosphatase activity using a phosphopeptide as the enzyme substrate. Comparable levels of free phosphate were also released by anti-Flag immunoprecipitates from P1 cells, transfected with either wtIDO or IDOH350A. The absence of either ITIM1 or ITIM2 significantly impaired release, which was virtually undetectable in the absence of both ITIMs. Therefore, our data suggested that IDO phosphorylation is necessary for binding and activating SHP-1 and SHP-2 phosphatases. This interaction determines regulatory effects - including impaired tumour antigen presentation - that were unrelated to tryptophan catabolism. Thus, this study documents a novel mechanism of immune modulation by metabolic pathways which have been very recently identified as possible innovative drug targets in immune suppression.

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Evidence for the role of PPAR-β/δ in the development of spinal cord injury *in vivo* and *in vitro* models

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Several lines of evidence suggest a biological role for peroxisome proliferator-activated receptor (PPAR-β/δ) in the pathogenesis of a number of diseases (Cuzzocrea et al. 2006). The aim of the present study was to evaluate the contribution of PPAR-β/δ in the secondary damage in experimental spinal cord injury (SCI) in mice. To this purpose, we used GW0742 which is a high affinity PPAR-β/δ agonist. Spinal cord trauma was induced by the application of vascular clips (force of 24 g) to the dura via a four level T5-T8 laminectomy (Genovese et al. 2006). SCI in mice resulted in severe trauma characterized by oedema, neutrophil infiltration, and production of inflammatory mediators, tissue damage, and apoptosis (Profyris et al. 2004). GW0742 treatment (0.3 mg/kg, i.p.) 1 h and 6 h after the SCI significantly reduced: i) the degree of spinal cord inflammation and tissue injury (histological score); ii) neutrophil infiltration (myeloperoxidase activity); iii) nitrotyrosine formation; iv) pro-inflammmaory cytokines expression; v) NF-κB activation; vi) iNOS expression; vii) apoptosis (TUNEL staining, FasL, Bax and Bcl-2 expression). Moreover, GW0742 significantly ameliorated the recovery of limb function (evaluated by motor recovery score). In order to elucidate whether the protective effects of GW0742 are related to activation of the PPAR-β/δ receptor, we also investigated the effect of a PPAR-β/δ antagonist, GSK0660, on the protective effects of GW0742. GSK0660 (1 mg/kg administered i.p. 30 min prior to the treatment with GW0742) significantly blocked the effect of the PPAR-β/δ agonist and thus abolished the protective effect (Hansen et al. 2001; Saluja et al. 2001).
Moreover, we have now studied the possibility to use organotypic slice cultures of spinal cord, cut in the longitudinal sagittal plane, to asses axonal growth and regeneration of neurons after damage induced by spinal cord injury (Batchelor et al. 2002).
Our results, *in vitro* and *in vivo* study, clearly demonstrate that GW0742 treatment reduces the development of inflammation and tissue injury associated with spinal cord trauma.

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Glycosaminoglycans characterization in neurodegenerative processes and new pharmacological strategies: *in vivo* and *in vitro* studies

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Proteoglycans (PGs) represent a diverse class of complex macromolecules and share a general molecular structure that includes a central core protein with a number of covalently attached carbohydrate chains, the glycosaminoglycans (GAGs). Each GAG is made up of repeating disaccharide units (hexuronic acid and N-acetyl hexosamine) and there are four classes of GAGs based on different chemical structures: chondroitin and dermatan sulfate (CS/DS); hyaluronic acid (HA); keratan sulfate (KS); heparin and heparan sulfate (HP/HS) (Hascall et al. 1970). In the central nervous tissue, the extracellular matrix forms a special matrix called perineuronal net characterized by several types of GAGs (Fox et al. 2002) with different patterns of sulfation and different biological properties and functions (Sugahara et al. 2003). Previous studies suggest that GAGs may play an important role in the pathogenesis and/or alleviation of neurodegenerative disorders, including Alzheimer's disease (AD) (Pollack et al. 1995; Dudas et al. 2008). My doctoral work has dealt with the fine characterization of the structure and the physico-chemical properties of GAGs involved in AD. The structural and chemical modifications of the perineuronal net in AD were studied; moreover, the possible neuroprotective effect of GAGs with different molecular weight and pattern of sulfation was investigated. An animal model of AD was obtained by the injection of a β-amyloid solution into striatum; after five days, the rat striata were collected for the characterization of GAGs by agarose gel electrophoresis, SAX-HPLC and fluorophore-assisted carbohydrate electrophoresis (FACE). The results showed that the intrastriatal injection of β-amyloid produced a significant decrease (-40.8%) in HA and an increase (+14.5%) in DS with a total charge density increasing of 14.9%. These results indicate that perineuronal network undergoes deep modifications in brain areas showing extra-cellular amyloid plaques. On the other hand, these data support the hypothesis that an increase in local production of sulfated GAGs might have a main role in reducing β-amyloid neurotoxicity. However, the mechanism underlying the neuroprotective action of GAGs has not yet been fully clarified. Several mechanisms have been proposed to explain how Aβ triggers neuronal death. In particular, some studies suggest that β-amyloid interacts directly with lipid membrane inducing lipid peroxidation and consequently reactive oxygen-induced damage. Other studies indicate that β-amyloid, binding to glutamate receptors, induces sustained elevation of intracellular Ca^{2+} levels. Increased oxidative damage and sustained Ca^{2+} levels lead to the generation of intracellular free radicals causing DNA fragmentation and apoptotic genes activation (Harkany et al. 1999). On the bases of these mechanisms, the hypothesis of GAGs protection on β-amyloid-induced neuronal damage was evaluated using *in vitro* models. The oxidative stress model was reproduced by blocking mitochondrial complex I with rotenone (30 μM). As a result of mitochondrial disruption, the cell cannot further synthesize ATP, free radicals are generated beyond the cell's capacity to buffer them, causing cell death. Our results showed that exposure of SH-SY5Y cells to rotenone for 24 h decreased cell viability (about -40%), measured by MTT assay. Pretreatment of the cells with different CS (bovine, porcine, squid, and ichthyic) at the concentrations of 10 and 50 μM, 24 h before and during the toxic stimulus, afforded significant cell protection. The maximum activity was observed with bovine CS (increase in cell viability of about 20% vs rotenone-treated cells) while no protection was observed with HA, the only one non-sulfated GAG. On the other hand, the excitotoxicity cell model was obtained exposing SH-SY5Y neuroblastoma cells to glutamate (60 mM) for 24 h. Preliminary data suggest that the bovine CS is able to counteract glutamate toxicity but further experiments are necessary. Overall, the results obtained in the present research allow proposing perineuronal net modifications as new molecular mechanisms underlying the pathogenesis of AD. Moreover, the identification of specific GAGs with neuroprotective effects might lead to the development of new drugs for the therapy of neurodegenerative diseases.

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In vivo effects of extra-virgin olive oil: long-term studies in animal models

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As the world population is increasingly aging, it is important to identify protective interventions aimed to reduce the deleterious effects of aging, such as the increased incidence of disabling diseases.
There is growing interest in finding dietary strategies capable of protecting from the deleterious effects of ageing on the brain, and antioxidants have been widely investigated in this respect. Among these, flavonoids have shown neuroprotective potential both *in vitro* and *in vivo* (Youdim et al. 2002).
The aim of the present work was to verify whether extra-virgin olive oil (EVOO), a food naturally containing phenolic antioxidants, has the potential to protect from the deleterious effects of aging. Experiments were conducted in rodents, subjected to high- or intermediate-calorie diets from adult to old age.
For the high-calorie studies, male Wistar rats were used and fed from age 12 months to senescence a 23% fat diet containing either corn oil, or EVOO with high or low phenol content. The measured endpoints were biochemical parameters related to oxidative stress and functional tests to evaluate motor, cognitive, and emotional behaviour. Under these experimental conditions, olive oil phenols did not exert major protective actions on locomotor and cognitive functions, but induced a tendency towards amelioration of motor coordination and higher mid-term survival. The main unexpected finding of this work was however an anxiety-lowering effect in the older animals upon treatment with olive oil rich in antioxidant phenols. This indicates an interesting and previously undetected effect of a diet containing an olive oil rich in phenols and suggests the possibility of modulating anxiety with supplements or natural foods rich in these compounds.
For the intermediate-calorie studies, male C57Bl mice were used and fed from age 12 months to senescence a 10% fat diet containing EVOO with high or low phenol content. An additional group fed EVOO with low phenol content plus resveratrol (22 mg/kg b.w. final dose) was used as positive control. The main findings obtained in behaviour were amelioration in the performance on the rotarod, indicating improvement in motor coordination, and an increase in muscular strength as measured in the grip strength test, brought about by the EVOO with high phenol content. Furthermore we investigated some oxidative damage parameters, such as antioxidant enzymes activity and lipid peroxidation, and some inflammation status markers. These data indicated that resveratrol and olive oil phenols share some health-promoting effects in aging rodents.

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Modulation of ATPase activity of rat small intestine membrane vesicles by novel N,N-bis(cyclohexanol)amine aryl ester derivatives

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Many tumor cells become resistant to commonly used cytotoxic drugs due to the overexpression of ATP-binding cassette (ABC) transporters. Pgp (MDR1, ABCB1) and MRP1 (ABCC1) have been demonstrated to pump a wide selection of the most commonly used cancer drugs (Fusi et al. 2006). Several generations of inhibitors of Pgp and MRP1 have been examined in preclinical and clinical studies. The main problems associated with the development of these drugs seem due to poor specificity, low potency, and interference with physiological functions (O'Connor 2007). Studies of the effects of MDR modulators on ATPase activity of the ABC transporter family are considered useful to get insight into the action mechanisms of these drugs. The effects of N,N-bis(cyclohexanol)amine aryl ester derivatives previously proven to be powerful inhibitors of Pgp (Martelli et al. 2009) on pump ATPase activity of brush border plasma membrane vesicles, prepared from homogenates of rat small intestine mucosa (Kessler et al. 1978), were evaluated.
ATPase activity was measured spectrophotometrically at 37°C using an ATP-regenerating system (pyruvate kinase and phosphoenolpyruvate), coupled to lactate dehydrogenase and NADH (Urbatsch et al. 1995), by monitoring NADH absorbance decay with time at 340 nm. Sodium azide, EGTA, and ouabain were added to the reaction medium to inhibit, respectively, H^+-, Ca^{2+}-, and Na^+/K^+-ATPases. NADH absorbance decay with time curve followed a polynomial equation and ATPase activity was calculated by the curve tangent at 1300 s.
In plasma membrane vesicles, MRP1, MRP2, and Pgp were present in detectable amounts. On the assumption of the existence of multiple binding sites on Pgp and sister proteins, both basal ATPase activity and ATPase activity stimulated by the substrate verapamil and epirubicin were assayed in the absence or in the presence of MDR-inhibitors. In the assay protocol used, however, ATPase activity was fully inhibited by low micromolar concentrations of Na_3VO_4 (IC_{50}=180 nM), thus indicating that it was totally ascribable to Pgp or MRPs. Verapamil and epirubicin modulated the basal ATPase activity, thus behaving like a substrate for the pumps. Both compounds, in fact, gave a typical bell shaped concentration-activation curve with 3 μM and 1 μM maximum effective concentrations, respectively. Overnight dialyzed vesicles were also prepared in order to remove low molecular weight endogenous substrates and then used to test epirubicin effect. In this case, basal activity was halved while that stimulated by epirubicin was unchanged. Finally the effects exerted by the trans/cis isomer MC260 was assessed. At concentration of 50 fM, MC260 inhibited the basal ATPase activity while it antagonized that stimulated by epirubicin with an IC_{50}=0.18 fM.
In conclusion, the results show that MC260 behaved like a potent and efficient inhibitor of ATPase activity, underlining the high affinity and specificity of the interaction of this new class of N,N-bis(cyclohexanol)amine aryl esters with MDR proteins-related ATP-ase.
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Polydeoxyribonucleotide (PDRN) restores blood flow and improves angiogenesis and wound healing in experimental model of ischemic skin wounds

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Chronic wounds represent a major public health problem. Ischemia, primarily caused by peripheral artery disease, represents a major complicating factor in the development and poor prognosis of chronic wounds (Sen 2009; Steinbrech et al. 1999). The purpose of this study was to assess the capacity of polydeoxyribonucleotide (PDRN) to improve wound healing (Montesinos et al. 1997) and angiogenesis (Galeano et al. 2008; Montesinos et al. 2004; Bitto et al. 2008) in a rat model of ischemic skin wounds. A total of 84 male Sprague-Dawley rats were used. Normal incisional wound and H-shaped double flaps were used as wound models. After the surgical procedures, the animals were randomized to receive PDRN (8 mg/kg/i.p., n=21 rats for each group) or vehicle (NaCl 0.9%/i.p., n=21 rats for each group). Rats were killed on different days (3, 5, and 10 days after skin injury) after the evaluation of skin perfusion by laser Doppler, and the wounded skin tissues were used to measure VEGF, HIF-1α and iNOS expression, as well as for histological analysis. Ischemic wounds showed a dramatically decrease of tissue perfusion compared with incisional wounds. A marked increase in blood flow was observed in ischemic wounds group treated with PDRN at 8 mg/kg after 3 days and gradually over time, with a complete recovery starting from day 5. Moreover, Western blot analysis showed that ischemia caused a marked increase in the VEGF, HIF-1α and iNOS expression in the ischemic wound at days 3 and days 5, compared to incisional wounds. The administration of PDRN further enhanced the expression of VEGF, HIF-1α, and iNOS in the ischemic wounds, at days 3 and 5, while the expression was barely detectable at days 10 after wound. These molecular data were confirmed by histological analysis. In fact, untreated ischemic wound showed a still incomplete reepithelialization, with a very low organized granulation tissue. In contrast, a complete reepithelialization and well-formed granulation tissue rich in fibroblasts oriented parallel to the epidermal layer were observed in the ischemic skin wound treated with PDRN. These results suggest that PDRN restores blood flow and improves angiogenesis and wound healing in experimental model of ischemic skin wounds and may be an effective therapeutic approach in improving healing in ischemic skin wounds.

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Study of interactions between ABC-transporters and metabolism in rat intestinal "precision-cut" slices

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Intestinal metabolism (phase I and/or phase II) and drug transporters (phase III) have been recognized as major physiological mechanisms that protect organisms from toxic compounds. Several phase III proteins, commonly known as ATP-binding cassette (ABC), play key roles in tissue maintenance by transporting metabolic waste and toxic chemicals out of cells (Benet et al. 1999). To study xenobiotic interactions with ABC proteins like multidrug-resistance protein (MDR) and MDR associated protein (MRP), intact cell systems are required. The aim of the present study was to set up an intestinal precision-cut slice technique to study the interaction between ABC-transporters and xenobiotic-metabolizing system.

Slices were prepared as described by De Kanter et al. (2005) and individually incubated in RPMI 1640 under 95% O_2 5% CO_2 atmosphere at 37°C in 12 well plates in the presence of 0.5 µM calcein AM and 10-100 µM of chlorpyrifos as transport inhibitor. The intracellular de-esterification product of calcein AM, calcein, was measured spectrofluorimetrically. The presence of chlorpyrifos increased the intracellular concentration of calcein in a time-dependent fashion (with a maximum value at 30 or 90 min incubation time at 50 µM and 100 µM, respectively) and promoted a concentration-dependent accumulation of calcein. Furthermore the presence of 100 µM ketoconazole, a CYP-dependent metabolism inhibitor, decreased the ABC-transporters inhibition promoted by chlorpyrifos when it was present in the medium up to 90 min incubation time. The presence of chlorpyrifos-oxon, the major cythorcome P450-dependent metabolite of chlorpyrifos, was detected in intestinal precison-cut slices by HPLC-MS analysis.

These data suggest the involvement of CYP-dependent metabolism in the inhibition of ATP-transporters promoted by chlorpyrifos. Furthermore the experiments indicated that the precision-cut intestinal slices are a reliable, simple, and fast system for evaluating xenobiotic interactions with ABC transporters. Together with data already appeared in the literature (Van de Kerkhof et al. 2007), these results indicate that the model is also suitable for studying phase I and phase II drug metabolism.

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Glucocorticoid dependent caspase-8 activation protects glucocorticoid-induced leucine zipper (GILZ) from proteasomic degradation

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Glucocorticoids (GCs) induce thymocyte apoptosis by protein synthesis and caspase activation. Glucocorticoid-induced leucine zipper (GILZ)is a protein transcriptionally induced by GCs and able to induce thymocyte apoptosis by activation of caspase-8 and caspase-3. In order to see if there is a cross-talk between GILZ and caspases, caspases activation of dexamethasone (DEX)-treated thymocytes was inhibited by specific caspase-8 or caspase-9 inhibitors. As already known, DEX-induced thymocyte apoptosis was inhibited by blocking the activation of either caspase-8 or caspase-3 but not caspase-9. In parallel on same groups DEX-induced GILZ expression, as evaluated by Western blot, was inhibited by caspase-8 but not caspase-9 inhibitors.
The GILZ mRNA analysis shows that, when inhibition of caspase-8 occurrs, the GILZ mRNA is normally transcribed after thymocyte DEX-treatment.
To see if the caspase-8 dependent inhibition of the expression of GILZ protein was a consequence of protein degradation, DEX-treated thymocytes in which caspase-8 activation was inhibited by its specific inhibitors, were also treated with two proteasome inhibitors (MG-132 or lactacystin), or with ALLN, an inhibitor of lysosomal enzymes. By inhibiting caspase-8 and proteasome, GILZ is re-expressed but this does not happen when lysosome are inhibited, showing that DEX-induced caspase-8 activation protects DEX-induced GILZ protein from its proteasomal degradation. Post-transaltional modification by ubiquitin plays a central role in targeting proteins for proteolytic degradation by the proteasome, although covalent binding of ubiquitin to proteins can also regulate localization and/or activities independent from proteolysis. In addition to ubiquitin, there are several ubiquitin-like proteins and one of them in particular (SUMO-1) has been shown to covalently modify a large number of proteins by an enzymatic machinery similar to the ubiquitination machinery. In some cases, SUMO and ubiquitin may directly compete for modification of the target lysines, thus inhibiting each other's binding to target proteins. We evaluated whether the caspase-8 activation-dependent protection of GILZ proteasomal degradation may be due to a caspase-8 driven sumoylation of GILZ that inhibits ubiquitination on the same lysine acceptor site. Analysis of the amino acid sequence of GILZ revealed the presence of a putative SUMO binding site. Therefore we tested whether SUMO-1 could associate with GILZ by using glutathione-S-transferase-GILZ pull-down assay. SUMO-1 bound GILZ with high affinity only on GC-treated thymocytes, but not in untreated cells or when GC-dependent activation of caspase-8 was inhibited by a specific inhibitor. In GC-treated thymocytes where caspase-8 was activated but caspase-3 activation was inhibited by a specific inhibitor, GILZ maintained the ability to bind SUMO-1. Thus, inhibition of caspase-8, but not caspase-3, activation abrogated the binding of SUMO-1 to GILZ.

CYPs and related nuclear receptors expression in response to a high fat diet in hepatic and extrahepatic tissues of pig

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Cytochrome P450 (CYP) is undoubtedly one of the most versatile enzyme systems, responsible for the metabolism of xenobiotics, but also for the biosynthesis and catabolism of endogenous substrates such as vitamins, hormones, prostaglandins, and fatty acids (Guengerich 1993). CYP activity is greatly influenced by a number of factors such as genetics, nutrition, and previous exposure to other chemicals, and can be also modulated by disease in both animal models and in humans (Ioannides et al. 1993). Atherosclerosis-induced cardiovascular and cerebrovascular diseases constitute a major cause of death in the western world, and major factors predisposing to the disease have been identified as high fat diet and smoking. In the latest years, pig has become of interest because it has been proposed as a new model for pharmacological and toxicological studies, and also for atherosclerosis (Thim et al. 2010). In the context of a wide and ambitious project involving many clinical researchers to find early markers of the atherosclerotic process, we investigated the expression and activity of the principal xenobiotic-metabolising CYP proteins in the hepatic and extrahepatic tissues of pigs subjected to a high fat diet. Five male castrated pigs of about 30-40 kg were utilized as controls and five were subjected for two months to 700 g/day of a diet containing 20% of lard, 4% of cholesterol, and 15% of sodium cholate, besides proteins and carbohydrates. Samples of liver, duodenum, heart, and coronary arteries were collected after killing the animals, and total RNA was extracted, retrotranscripted and amplified by PCR using specific primers for many pig CYPs (such as CYP2B22, 2Cs, 3As, 2E1, 2J, 4A), for nuclear receptors involved in their modulation (CAR, PXR, PPARα, LXRα), as well as for CYP7A1 and CYP27A1, which are involved in cholesterol metabolism. Furthermore, hepatic, intestinal, and cardiac microsomes were prepared in a standard way to investigate the activity of the main CYP subfamilies through a wide range of marker reactions: ethoxyresorufin-O-deethylase for 1A subfamily, etoxytrifluoromethylcoumarin O-deethylase and 9-anthraldehyde oxidase for 2B subfamily, p-nitrophenol hydroxylase and aniline hydroxylase for CYP2E1, paclitaxel hydroxylase, tolbutamide hydroxylase, diclofenac 4'-hydroxylase, and metoxytrifluoromethylcoumarin O-demethylase for 2C subfamily, benzyloxyquinoline debenzylase and erythromycin N-demethylase for 3A subfamily, lauric acid hydroxylase for 4A subfamily. The hydroxylation of testosterone was also evaluated. Finally, immunoblot experiments, carried out using antibodies anti-CYP2C11, 2E1, and 4A1 of rat, were performed. The results of the activities showed a decreasing trend in liver and duodenum for the marker of CYP2C and 2B subfamilies. Instead, p-nitrophenol hydroxylase activity, a marker of CYP2E1, was increased in liver microsomes of high fat diet treated-pigs, in agreement with what is known in rodents (Murray 2006). No changes was evident in the heart activities, in agreement with experiments carried out in rabbits administrated with 1% cholesterol diet for 8 weeks (Irizar et al. 1998). The 15β-hydroxylation of testosterone (mainly due to CYP3A1 in rodents) was increased in liver microsomes of treated pigs, but the others tested CYP3A marker activities were not modulated. On the contrary, the oxidation of testosterone in the position 17 was decreased by the high fat diet. Nevertheless, neither the transcriptional analysis, nor the immunoblot experiments showed a significant modulation of the tested CYPs. The expression of CYP and receptor genes in the coronary arteries is in progress. In general, while in rodents the administration of a high fat diet, even lighter than the one utilized for this study, results in a profound modulation of drug metabolising enzymes, in pig this effect is very weak. This could be due: a) to the duration of the diet, maybe too short to appreciate chronic adaptations; b) to the youth of the animals, which render pigs more adaptable; c) to the nature of the pig species, omnivorous and thereby more refractory to diet-dependent effects on CYP expression.

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Engagement of toll like receptor-2 by *herpes simplex* virus–1 contributes to neuropathogenesis of mouse enteric nervous system

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Herpes simplex virus type 1 (HSV-1) appears to be a potential candidate pathogen involved in gastrointestinal motor disorders (GIMD) (Gesser et al. 1996). Recently, we have shown that HSV-1, orally inoculated to laboratory animals, infects the enteric nervous system (ENS) and determines an altered neuromuscular function and an increase of cytokines in longitudinal smooth muscle-myenteric preparations (Zoppellaro et al. 2009; Brun et al. 2010). Sensing of human herpes viruses by toll like receptor-2 (TLR2) results in the activation of an inflammatory response and although this is usually a protective mechanism, in the case of HSV-1 it has been shown that the TLR2-mediated cytokine response contributes to neuropathogenesis in the host central nervous system. *In vitro* contractility studies performed in our laboratory have shown that receptor and not-receptor mediated responses were altered in wild type mice and these impairments were even more pronounced in TLR2-deficient mice supporting an involvement of TLR2 in the anti-viral responses and GIMD onset (Kurt-Jones et al. 2004). The aims of the present study were to: i) assess the effect of HSV-1 infection on the neurochemical phenotype of ENS; ii) evaluate the critical role of TLR-signalling in ENS neurodysfucntion induced by HSV-1 infection. Wild type and TLR2 deficient mice (male, C57Bl/6; 20±2 g) were inoculated with HSV-1 intranasally and, after 4 weeks (W), intragastrically. After 1-8 W, HSV-1 infection was determined in the brain and in freshly isolated myenteric ganglia by molecular analysis. The neurochemical phenotype of the ENS was analyzed with antibodies against Hu C/D, (somata neuronal marker), S-100β (glial marker) and peripherin (process and somata neuronal marker), and with acetylcholinesterase staining in ileum whole mount preparations. The morphoquantitative analysis of myenteric ganglia was performed in 5 random ganglionic fields for each mouse using confocal microscopy and Nis Elements vs. 3.0 as image analysis system. The mRNA expression of acetylcholinesterase enzyme and TLR2 receptor was assessed by quantitative RT-PCR analysis. A significant reduction of the ganglionic areas in the myenteric plexus was found in WT mice 1 W (-36%) and 8 W (-21%) postinfection (PI). S-100β positive glial cells increased from 22±3 to 51±5 in sham and 6 W PI, respectively. In the ENS the Hu C/D labelling was significantly decreased at 1 W PI (-37%) and further reduced at 6 W and 8 W after HSV-1 infection. Quantitative RT-PCR analysis showed TLR2 mRNA overexpression resulting in two-fold and six-fold increases over sham at 1 W and 4 W PI, respectively, in isolated myenteric ganglia, suggesting an early involvement of innate immune responses during HSV-1 infection of the ENS. In sham TLR2 KO mice, the myenteric ganglia area was significantly reduced only at 8 W PI, when the staining of S-100β positive glial cells and Hu C/D positive neurons started to vanish. By performing peripherin staining we found an astonishing neurofilament disorganization (e.g. irregular distribution and fragmentation) at 1 W, 6 W, and 8 W PI in WT mice whereas in TLR2 KO mice it was detected only at 8 W PI. A reduced staining of acetylcholinesterase-positive neurons, large fibers and small fibers, was evident at 1 W, 6 W, and 8 W PI in WT mice whereas an overexpression of acetylcholinesterase mRNA was found at 2 W and 4 W PI. In TLR2 KO mice the neural distribution of the enzyme did not appear to be affected by HSV-1 infection. These results provide evidence that HSV-1 induced altered neuromuscular function is caused by a significant ENS injury. Moreover, HSV-1 interaction with TLR2 contributes to neurodysfunction of ENS in the early stages of the infection, highlighting the potentially detrimental effects of TLRs responses on enteric nerves.

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Observational study comparing the safety of typical and atypical antipsychotic drugs in patients affected by dementia with behavioural and psychological symptoms of dementia

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Dementia is a clinical syndrome characterised by cognitive impairment, which represents a decline from previous level of functioning, and is associated with impairment in functional abilities and, in many cases, behavioural and psychiatric disturbances (BPSD) such as aggression, agitation, hallucinations, psychosis, depression, and wandering. The prevalence of dementia increases between the seventh and ninth decade from 20% to 30% and it is estimated that in Italy there are approximately 765,000 patients with dementia. Typical and atypical antipsychotic are often prescribed to manage BPSD in elderly, although such prescriptions represent off-label prescribing and, to date, there are a few data from clinical trials which support their efficacy and safety (Wang et al. 2005). Moreover, there are concerns about the increased risk of cerebrovascular adverse events (AEs) and mortality associated with antipsychotic treatment (Schneider et al. 2006). These findings have prompted health authorities to take restrictive regulatory actions. Based on these evidences it was necessary to conduct a prospective cohort study to: i) evaluate atypical and typical antipsychotic use among patients with dementia and BPSD; ii) estimate whether the mortality and incidence of serious AEs associated with atypical and conventional antipsychotics; iii) compare safety profile of both types of treatments; iv) define risk factors influencing the appearance of AEs; and v) describe covariates distribution among population based study. An observational cohort study is being carried out in patients with dementia and BPSD treated with either typical or atypical antipsychotic drugs. All subjects, non-institutionalised, were enrolled at 91 specialized centers (SC) identified by Campania Region as experts in the diagnosis and treatment of dementia. All data are collected using an ad-hoc questionnaire and all AEs and clinical outcomes are carefully monitored. Our preliminary data showed that patients with dementia and BPSD are 2841 (1908 females; 67.2%). 1513 (53.2%) patients are affected by dementia of the Alzheimer's type, 890 (31.0%) by vascular dementia, and 140 (4.9%) by mixed dementia. Hallucinations (23.0%), aggression (22.0%), agitation (21.0%), and delirium (16.0%) are the most common BPSD. The most frequently prescribed antipsychotics are quetiapine (52.0%), risperidone (22.5%), and olanzapine (18.9%). At the time of enrolment, most patients had at least one of the following risk factors: hypertension (43.0%), vasculopathy (25.0%), diabetes (12.0%), cardiopathy (12.0%), and dyslipidemias (8.0%). The most frequent concurrent therapy are antidepressant medications (31.7%) followed by anticoagulant (12.2%), antiplatelet (8.7%) and antidiabetic drugs (6.1%). During the monitoring period 84 AEs were observed and the most frequent of them were drug failure (55.3%) and extrapyramidal symptoms (9.5%). We also observed 103 deaths (3.6%), all such patients receiving atypical antipsychotics. The results of this study will supply elements in the determination of the risk-benefit profile of conventional and atypical antipsychotic drugs in non-institutionalised patients affected by dementia and BPSD.

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Pattern of triptan use in a sample of the Italian general population

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My first year doctoral work was based on a study about the utilization pattern of triptans (5-$HT_{1B/D}$ agonists), a specific class of drugs for the treatment of acute migraine episodes. Migraine is an episodic pain disorder affecting approximately 14.7% of the population and it is over 2 times more common in women than in men (Stovner et al. 2010). Although clinical trials showed a favourable risk-benefit profile (Pascual et al. 2007) under appropriate criteria of use, pattern of triptan consumption in real life settings has been poorly examined.
The aim of the research was to observe triptan users in terms of prevalence and pattern of utilization, and to verify the presence of concomitant cardiovascular drug prescriptions that could identify patient with contraindications to triptan use (i.e. stroke and myocardial infarction). To investigate this concern, all patients receiving their first triptan prescription ("new users") in January-December 2007 were selected from the Emilia Romagna drug prescription database and prospectively observed for 12 months. Patients were divided into 3 categories of frequency: occasional users (1-12 dosage units/year), normal users (13-30 dosage units/year) and strong user (>30 dosage units/year). Analyses by gender and age were also performed.
Among 16,811 new users, 73.7% took up to 12 dosage units/year (12,387 subjects, of which the 55% was one-prescription users), only 14.1% used 13-30 dosage units/year, and 12.2% received more than 30 dosage units/year. Women were 75% and the majority of patient was aged between 20-50 years old. Every month, new users accounted for about 14% of all subjects receiving a triptan prescription; no significant differences among months were detected.
Concerning triptan use in patients with cardiovascular risk (Jamieson 2002), we found a concomitant therapy with cardiovascular drugs in 36.6% of all new users. Moreover, cardiovascular co-prescriptions markedly increased with increasing age, reaching the 17.3% (204/1179) in older new users (>65).
These findings are confident with previous drug utilization studies (Panconesi et al. 2008) showing that the majority of patients discontinued triptan use after their first prescription and subjects who continued the treatment in the subsequent 12 months used a low number of dosage units.
Despite the known increased risk of vasoconstrictive complications related to the pharmacological mechanism of these antimigraine drugs, our data suggest that a large percentage of triptan users could have a basal cardiovascular risk.

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Resistance artery mechanics and composition in angiotensin II-infused mice: effects of cyclooxygenase-1 inhibition

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Angiotensin (Ang) II is greatly implicated in the development/progression of structural alterations (vascular remodelling) in small-resistance arteries (Touyz 2005). Increased media to lumen ratio (M/L), which characterizes vascular remodelling, can result from a reduced outer diameter, which narrows the lumen without net growth (eutrophic remodelling), or from a thicker media encroaching on the lumen (hypertrophic remodelling) (Schiffrin 2004). Another hallmark of Ang II-induced structural abnormalities is represented by changes in the mechanical properties of arteries, with particular regard for increased stiffness (Intengan et al. 2000). Endothelial dysfunction secondary to Ang II infusion is partly dependent on COX-1 pathway. Ang II is associated with COX-1 overexpression and activity, which in turn produces a contracting prostanoid endowed with agonist activity on TP receptors (Virdis et al. 2007). Recent data documented a predominant role of COX-1 derived prostanoids in the development/progression of vascular atherosclerotic process (Praticò et al. 2001).
The aims of this study were to evaluate whether COX-1 derived prostanoids, acting on TP receptors, contribute to changes in vascular mechanics and extracellular matrix (ECM) components, which characterize the vascular remodelling of mesenteric resistance arteries elicited by Ang II. For this purpose, we employed selective COX isoforms inhibitors, chronically administered to Ang II-treated mice to wild type (WT), and homozygous mice carrying a targeted disruption of the COX-1 gene ($COX\text{-}1^{-/-}$).
Experiments were carried out on WT and $COX\text{-}1^{-/-}$ mice, treated with vehicle or Ang II (400 ng/kg/min, s.c.) (Brassard et al. 2005). Ang II-infused WT mice also received SC-560 (COX-1 inhibitor, food), DFU (COX-2 inhibitor, food) or SQ-29548 (TP receptor antagonist, i.p.). After two weeks, mesenteric vessels were exposed to intraluminal pressures (3-140 mmHg, pressurized myograph) to determine mechanical properties (Neves et al. 2004). Collagen type I and fibronectin were evaluated with immunostaining, and elastin histochemistry was performed by staining sections with the Taenzer-Unna orcein method. Systolic blood pressure (SBP) was significantly increased by Ang II infusion as compared to controls, and not modified by any test drug. In $COX\text{-}1^{-/-}$ mice, the Ang II-induced SBP increment was less evident. In WT mice, M/L ratio and media cross-sectional area were increased by Ang II, reversed by SC-560 or SQ-29548 ($P<0.01$), but unaffected by DFU. Ang II induced stiffer vessels ($P<0.01$), as documented by the shift to the left of the stress-stain curve. This effect was ameliorated by SC-560 or SQ-29548, but not modified by DFU. $COX\text{-}1^{-/-}$ mice showed normal vessel elasticity, which was not affected by Ang II. Collagen and fibronectin depositions and decreased elastin content were induced by Ang II ($P<0.01$), reversed by SC-560 or SQ-29548, but unaffected by DFU. In $COX\text{-}1^{-/-}$ mice, Ang II did not change collagen, fibronectin or elastin contents. In WT mice, Ang II induced COX-1, decreased COX-2 expression (RT-PCR and Western blot analysis) and enhanced the vascular release of 6-keto-$PGF_{1\alpha}$, which was prevented by COX-1 blockade.
In conclusion, Ang II-induced alterations of resistance arteries in structure, mechanics and ECM composition were prevented by COX-1 inhibition and TP receptor antagonism, indicating that some actions of Ang II on vascular remodelling are mediated by COX-1-derived prostanoid(s), including prostacyclin, activating TP receptors. These results strengthen the concept that COX-1 products are of importance in the pathophysiology of atherogenesis, and they can have important clinical relevance. Indeed, the observation that a COX-1-derived prostanoid is significantly involved in Ang II-induced vascular remodelling can be an explanation for the increased cardiovascular risk in patients treated with selective COX-2 inhibitors. It can be tentatively proposed that, in the future, pharmacological strategies aimed at preventing the activation of TP receptors should be taken into consideration to counteract the progression of vascular atherosclerosis, particularly in those forms of human hypertension characterized by a marked activation of the renin-Ang-aldoterone system.

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Survival from coma induced by an intentional 36 g overdose of extended-release quetiapine

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Quetiapine is a second-generation antipsychotic drug approved for the treatment of bipolar disorders and schizophrenia. Acute quetiapine overdose is rare in preclinical trials and it has long been deemed safer than other antipsychotic agents (Hustey, 1999). The most frequent symptoms of quetiapine overdose are tachycardia, agitation, hypotension, drowsiness, unconsciousness, and QT prolongation. Post-marketing reports of quetiapine overdose resulting in coma are rare, but quetiapine overdose can lead to coma more frequently than other antipsychotics (Ngo et al., 2008). To our knowledge, there are no reports of fatalities induced by more than 36 g quetiapine (Müller et al., 2009). Here, we describe the first case of coma induced by intentional ingestion of 36 g of quetiapine fumarate extended-release (XR). A 34-year-old woman with chronic schizophrenia was in coma (Glasgow Coma Scale of 9) after ingestion of 36 g of quetiapine fumarate XR for attempted suicide. The woman's clinical conditions rapidly deteriorated and she collapsed unconscious. She had been addicted to drugs (cannabis, heroin, LSD, cocaine, and crack) since adolescence. HIV infection was diagnosed when she was 18 years old and HCV (1a genotype) two years later. Her usual medication was lorazepam 2.5 mg/day, quetiapine 600 mg/day, tenofovir 300 mg/emtricitabine 200 mg/day, lopinavir 100 mg/ritonavir 25 mg/day, and raltegravir potassium 434.4 mg/day. On admission to the emergency department her blood pressure was 107/57 mmHg, heart rate was 70 beats/min and respiratory rate 12/min. Urinalysis was negative for tetrahydrocannibinol, opioids, methadone, cocaine, and amphetamine. Drug screening via immunoassay was positive for quetiapine. There were no indications that the patient had ingested anything other than quetiapine. The comatose state was attributed to drug toxicity and normalized with supportive care. Spontaneous breathing was restored in about 36 h. Antiretroviral therapy was re-instated 2 days after the adverse event. After 8 days, she was discharged and a week later she contacted the Center for Psychotic Disorders (Department of Psychiatry, Second University of Naples) for an appointment and she was immediately seen in our outpatient unit. At that time, she reported having experienced tiredness, somnolence, and dyspnea on effort after discharge from the hospital; these complaints resolved within a few days. After 4 months, she was prescribed quetiapine XR tablets (300 mg/day) and blood biochemistry and EKG findings were not significantly changed. She now regularly attends the follow-up visits at our outpatient unit. Quetiapine is becoming an increasingly important psychotropic drug in overdose because of an increased number of prescriptions in a population prone to overdose (Isbister, 2009). The severity of the effect of quetiapine overdose has not been associated with a high serum concentration of the drug or with the reported ingested dose (Hunfeld et al., 2006). In fact, death can result from quetiapine overdose at a concentration lower than 36 mg (Fernandes, 2002). In our case, recovery from an extreme oral overdose of quetiapine may be attributed to the absence of underlying cardiac disease, early intubation for airway protection, and the rapid transfer to an Intensive Care Unit.

Given the widespread use of quetiapine and the lack of information about its range of toxicity, this case report reinforces the importance of closely monitoring patients taking quetiapine, and helps to better define the safety of this drug.

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In vitro and *in vivo* pharmacological characterization of neuropeptide S receptor ligands

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Neuropeptide S (NPS) is the last neuropeptide identified via reverse pharmacology techniques. NPS selectively binds and activates a previously orphan GPCR, now named NPSR, producing intracellular calcium mobilization and stimulation of cAMP levels. Biological functions modulated by the NPS/NPSR system include anxiety, arousal, locomotion, food intake, learning and memory, pain, and drug addiction. In our laboratories we provided further evidence that NPS injected supraspinally in mice acts as a stimulatory anxiolytic. In fact, in the mouse righting reflex (RR) test, NPS (0.01-1 nmol, i.c.v.) was able to reduce in a dose dependent manner the percent of animals losing the RR in response to diazepam (15 mg/kg i.p.) and their sleeping time. Furthermore, NPS in the same range of doses caused a significant increase in locomotor activity (LA) in mice. These effects were associated with a clear anxiolytic-like action elicited by NPS in the mouse elevated plus maze test and stress-induced hyperthermia assay. Thus NPS evokes a unique pattern of behavioural effects: stimulation associated with anxiolysis (Rizzi et al. 2008). To deeply investigate the biological role of the NPS/NPSR system pharmacological (i.e. selective NPSR antagonists) and biological (i.e. receptor knockout animals) research tools are needed. In collaboration with the medicinal chemistry group of the University of Ferrara, in the context of structure-activity studies focussed on position 5 of NPS sequence, we identified and characterized *in vitro* and *in vivo* the first NPSR antagonist: [D-Cys(tBu)^5]NPS. *In vitro*, in HEK293 cells stably expressing the mouse NPSR (HEK293$_{mNPSR}$), [D-Cys(tBu)^5]NPS up to 100 μM did not stimulate calcium mobilization but was able to counteract in a competitive manner the stimulatory action of NPS (pA$_2$ 6.44). *In vivo*, in RR test, [D-Cys(tBu)^5]NPS at 10 nmol was inactive *per se* but dose dependently antagonized the arousal-promoting action of NPS 0.1 nmol (Camarda et al. 2009). In a further study another NPSR antagonist was identified: [D-Val^5]NPS. [D-Val^5]NPS acted *in vitro* as a pure NPSR antagonist, with a pK$_B$ of 6.54 in inhibition experiments. *In vivo*, in LA test, [D-Val^5]NPS at 10 nmol completely blocked the stimulatory effect evoked by NPS (Guerrini et al. 2009a). In a follow-up study, the most potent NPSR antagonist [^{t}Bu-D-Gly^5]NPS was identified. *In vitro*, [^{t}Bu-D-Gly^5]NPS did not stimulate calcium mobilization but blocked the stimulant action of NPS with a pK$_B$ of 7.06 (Guerrini et al. 2009b). *In vivo*, in RR assay, [^{t}Bu-D-Gly^5]NPS (0.1-10 nmol i.c.v.) was inactive *per se* but dose dependently antagonized the arousal-promoting action of NPS 0.1 nmol. Similarly in the LA assay [^{t}Bu-D-Gly^5]NPS (0.1-10 nmol i.c.v.) was inactive *per se* but was able to counteract the stimulatory effect evoked by 0.1 nmol NPS in a dose dependent manner. SHA 68 is a non peptidic NPSR antagonist identified by Takeda researchers and characterized pharmacologically *in vitro* and *in vivo* by Okamura (Okamura et al. 2008). In our laboratories we further investigated the pharmacological profile of SHA 68 *in vitro* and *in vivo*. *In vitro* SHA 68 was inactive *per se* up to 10 μM while it antagonized NPS-stimulated calcium mobilization in a competitive manner showing a pA$_2$ value of 8.06. *In vivo*, in the mouse RR assay, SHA68 50 mg/kg i.p. fully prevented the arousal-promoting action of the NPS 0.1 nmol. In LA experiments, SHA 68 50 mg/kg i.p. was able to counteract the stimulant effects elicited by NPS 0.1 nmol. Instead, the anxiolytic-like effects of NPS 0.1 nmol in mouse open field test were slightly reduced by SHA 68 (Ruzza et al. 2010). Collectively these data demonstrated the exclusive involvement of NPSR in the arousal promoting and locomotor stimulant effects of NPS. Finally, we backcrossed on the CD-1 strain the NPSR knockout (NPSR(-/-)) mice originally generated on the 129S6SvEv genetic background. A first study on these mice demonstrated that NPSR(+/+) mice and NPSR(-/-) mice are similarly sensitive to the hypnotic effects of diazepam. However, the arousal promoting action of 1 nmol NPS could be detected in NPSR(+/+) but not in NPSR(-/-) mice (Camarda et al. 2009). In the next months we will deeply characterize the NPSR(+/+) and NPSR(-/-) mice phenotype in different behavioural assays. In conclusion, the findings obtained from parallel pharmacological and knockout studies will be crucial for investigating the biological functions regulated by the NPS/ NPSR system and ultimately for identifying the potential indications of NPSR selective agonists and antagonists.

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Amyloid-β peptide (Aβ) and Alzheimer's disease: is endothelial-derived PGE_2 involved in barrier alteration due to Aβ?

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Alzheimer's disease (AD) is a well known age-related pathology characterized by deposition of amyloid-β peptide (Aβ) in brain parenchima and in the vessel wall. Aβ is derived by proteolysis of APP (amyloid precursor protein), through amyloidogenic pathway due to the action of β and γ secretases. The initial deposit of the peptide causes degeneration of vascular smooth muscle cells, pericytes, and endothelial cells (EC) which compromises the blood brain barrier (BBB) integrity and functionality. Brain capillaries are made up of non-fenestrated EC that are connected each other with tight junctions (TJ).
During previous years of my doctorate my interest was focused on characterizing the interplay between endothelium and vascular smooth muscle cells in response to Aβ challenge. In the course of this year I investigated the role of arachidonic acid (AA) cascade signalling pathway in this context.
The endothelial surface challenged by Aβ deposition stimulates the inflammatory cells to migrate and to pass through the BBB which triggers signal transduction cascade leading to reduction of TJ proteins and BBB leaking. Prostaglandin E_2 (PGE_2), the level of which is elevated in the brain during various inflammatory states, is a key mediator of BBB breakdown. PGE_2 is the pro-inflammatory end product of AA pathway. produced starting from AA through the subsequent action of cyclooxygenases (COX-1 and COX-2) and PGE-synthases (microsomal PGES1 and 2, cytosolic PGES). In particular COX-2 and mPGES1 are inducible, while the others are constitutive.
Several epidemiologic studies suggest that long-term use of non-steroidal-anti-inflammatory drugs (NSAIDs) may protect against AD even if the biological mechanism of this protection is not well understood (Imbimbo 2009). Furthermore the expression pattern of the enzymes involved in PGE_2 synthesis was investigated in AD patients brain and compared with age-matched controls, revealing no differences in COX-1, mPGES2 and cPGES levels between AD end-stage and control brain tissues, while COX-2 and mPGES1 expression was significantly elevated in AD patients brain tissue compared to control ones (Chaudry et al. 2008; 2009; 2010).
In this study the activity of Aβ was evaluated *in vitro* on human brain microvascular endothelial cells (HBMECs) to assess if the endothelial permeability alteration, measured in terms of downregulation of the TJ protein occludin, was linked to upregulation of both COX-2 and mPGES1 and to the over-production of PGE_2.
Aβ was administered for 6 and 18 h at different concentrations (from 1 nM to 10 μM). Results showed that expression of constitutive enzymes (COX-1, mPGES2, and cPGES) evaluated by Western blotting was not significantly affected upon Aβ administration. Conversely, COX-2 resulted upregulated in a time- and dose-dependent fashion. Unexpectedly mPGES1 expression was not altered after Aβ challenge except at 10 μM concentration, maybe due to its high basal levels found in this cell line. This conclusion was confirmed by high basal production of PGE_2, which did not increase in response to Aβ. With regards to occludin expression, also evaluated by Western blot, we observed its downregulation following both Aβ and AA (natural COX-2 substrate) administration.
Our findings suggest that Aβ lowers endothelium permeability in terms of occludin downregulation, but this modification seems not due to PGE_2 action. These data suggest that other pathways complementary to PGE_2 may control Aβ-induced EC permeability alterations.

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Study of the effects of repeated lithium or carbamazepine treatments on a model of mania in rats

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Long-term lithium or carbamazepine administration produces in rats behavioural and neurochemical effects analogous to those observed after long-term stress exposure, with some intriguing differences between the two compounds. Both induced escape deficit and decreased dopamine output in the shell portion of the nucleus accumbens (NAcS). We have tested the possible antimanic effect of both lithium and carbamazepine in morphine-sensitized rats. Morphine-sensitized rats present intense resilience to unavoidable stress-induced behavioural deficits, and are used as a model of mania. Chronic lithium or carbamazepine administration abolished stress resistance in morphine-sensitized rats without modifying the condition of behavioural sensitization, thus conferring predictive validity to the model.
Morphine sensitization in rodents is associated with a decreased threshold in μ-opioid receptor excitability in the striatal areas, and μ-opioid receptor stimulation in the NAcS plays a central role in evaluating the rewarding value of sweet food (Di Chiara et al. 2002). Since mania is often characterized by episodic excess in alcohol and food consumption, we hypothesized that morphine-sensitized rats might have a low threshold to sweet taste palatability and, thus, I studied the responsiveness of morphine-sensitized rats to palatable food exposure. Exposure to palatable food is used in experimental protocols that evaluate animal's hedonic competence since it elicits consistent behavioural and neurochemical responses (Danielli et al. 2010). Moreover, in these experiments only non food-deprived rats were used since in these animals the emotional value of a palatable food has a prevalent hedonic component. Rats are very fond of vanilla sugar (VS) and consumption of 4-5 small VS pellets increases the dopaminergic output in discrete prefrontal mesolimbic areas, in particular in the NAcS (Gambarana et al. 2003). Moreover, non food-deprived rats consistently acquire an instrumental behaviour based on the reinforcing properties of VS pellets, the earning of which is made contingent on the choice of one of the two divergent arms of a Y-maze (VS sustained appetitive behaviour, VAB). Thus, I compared the effects of repeated carbamazepine or lithium administration on the NAcS dopaminergic response to VS consumption and on the competence to acquire VAB in non food-deprived control and morphine-sensitized rats. Carbamazepine-treated control rats showed a reduced dopaminergic response in the NAcS to a palatable meal such as VS pellets, and no competence to acquire VAB; whereas control rats chronically treated with lithium showed intense dopaminergic response to VS consumption, and easily acquired VAB (Gambarana et al. 2001). Thus, at variance with lithium, carbamazepine seems to be endowed with anhedonic activity. In morphine-sensitized animals, repeated carbamazepine administration reduced but did not abolish the dopaminergic response to VS pellets consumption, although did not disrupt the competence to acquire VAB. Thus, carbamazepine treatment was able to reduce the strong hedonic impact of VS in sensitized rats (in terms of increases in NAcS dopamine levels), but did not impair their competence to operate in order to obtain a sweet reward. Repeated lithium treatment did not modify the dopaminergic response to VS consumption nor the competence to acquire VAB in morphine-sensitized rats (Gambarana et al. 2010). These results support the validity of the condition of morphine sensitization as an experimental model of mania and although lithium and carbamazepine showed a similar efficacy in reinstating the sensitivity to stress in sensitized rats, the two mood stabilizers showed remarkable differences in the way they affected the response to a natural reward, a sweet palatable food, both in control and sensitized rats.

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Pro-angiogenic activity of urotensin-II on cultured human vascular endothelial cells

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Urotensin-II (U-II) is a cyclic peptide, originally isolated from the urophysis of the goby *Gillichthys mirabilis*. The human form of U-II (hU-II) is a cyclic undecapeptide. Mature hU-II is synthesized from a large precursor molecule, the prepro-U-II, whose mRNA has been found in many tissues. U-II has been identified as the endogenous ligand of a specific high-affinity receptor, recently identified as the orphan receptor GPR14 (Ames et al. 1999) which has been renamed urotensin receptor (UT). The human UT isoform belongs to the class A superfamily of G-protein-coupled receptors. The principal physiological role of U-II in mammals is in the cardiovascular system, where it exerts a potent systemic vasoconstrictor and hypertensive effect and several lines of evidence suggested that U-II might be involved in the pathophysiology of the cardiovascular system (Douglas et al. 2000). On endothelial cells (ECs) of animal origin, U-II has also been shown to exert a clearcut pro-angiogenic effect (Spinazzi et al. 2006), but few experimental data are presently available clarifying the effect of U-II in human endothelium.
Thus, in the present study *in vitro* models based on human vascular ECs directly isolated from different normal human vessels, including saphenous vein (HSVEC), jugular vein (HJVEC), umbilical vein (HUVEC), and aorta (HAEC) have been used to further evaluate the angiogenic properties of U-II.
RT-PCR and ICC analyses indicated that UT was expressed by all the human ECs considered, while the expression of U-II resulted heterogeneous; in fact, the peptide was detectable in HAEC and HUVEC only. When tested in the Matrigel assay all the investigated ECs exhibited a strong angiogenic response to the peptide, with the formation of a meshwork of capillary-like structures of increased density and complexity when compared to the unstimulated condition. The effect was comparable to that of FGF-2 and was counteracted by palosuran, a specific antagonist of UT, indicating that they were triggered by the binding of U-II to its receptor. Interestingly, in ECs derived from adult vessels this activity was not associated with a proliferogenic effect. On the contrary, U-II induced a moderate but significant increase of cell proliferation in HUVEC.
Experiments performed in the presence of specific inhibitors of various steps of the signalling cascade showed that the U-II induced self organization of the cells in capillary-like structures is PKC dependent and involves the activation of the ERK1/2 transduction pathway. Western blot analyses on the phosphorylated forms of these kinases provided further support to this finding. Interestingly, the pharmacological inhibition of PI3K hindered the capacity of U-II to induce a pro-angiogenic effect on HUVEC, indicating that the PI3K/Akt pathway is also involved in regulating the process.
It has also to be observed that some of the signalling pathways activated as a consequence of the binding of U-II to UT can, in principle, be started in several ways. In fact GPCR can also indirectly activate them by inducing the synthesis and release of growth factors. In this respect, the stimulation of HUVEC with U-II for 24 h, induced AM, ET-1, and VEGF expression. All these factors are characterized by well known pro-angiogenic properties. Moreover, in human ECs VEGF gene silencing decreased the pro-angiogenic action of U-II when tested in the Matrigel assay, further confirming that the activity of the peptide could be significantly mediated by VEGF.
Altogether, the results of the present study suggest that U-II, in addition to regulating cardiovascular function, also exerts a direct action on the development and remodelling of the vascular network.

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Interaction between estrogen receptors and metabotropic glutamate receptors 1 in the neuroprotective effects of estrogen against β-amyloid toxicity

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Estrogen exerts its numerous effects, including those at the central nervous system, mainly through interaction with intracellular receptors, estrogen receptor α (ERα) and β (ERβ). This leads to the classical genomic action of estrogen that causes modulation of gene expression. ERs localized at the plasma membrane have also been described and their activation involves rapid signalling transduction mechanisms (Micevych et al. 2009). It has been suggested that membrane ERs bind and activate G-protein-coupled receptors using their signalling machinery to produce their rapid effects. Specifically an interaction between ERs and different metabotropic glutamate (mGlu) receptors has been demonstrated (Micevych et al. 2008). We have focused our attention on mGlu1, a Gq-linked receptor whose activation produces stimulation of phospholipase C and ensuing control of intracellular calcium mobilization (Bruno et al. 2001). An interaction between ERα and mGlu1 receptor has been demonstrated in hippocampal and hypothalamic neurons as well as in cultured astrocytes. To analyze the involvement of mGlu1 receptor in the neuroprotective effect of estrogen we have used mixed cultures of cortical neurons prepared from E17 rat embryos and maintained under conditions allowing the growth of both neurons and glia. Treatment with the β-amyloid (Aβ) active fragment, Aβ25-35 (25 µM), for 24 h caused a marked increase of neuronal death (over 100% above control), as assessed by cell counting after labelling with trypan blue, a dye excluded by viable cells. As expected, Aß25-35 had no effect on astrocyte viability. Pre-treatment with 10 nM 17 β-estradiol (17 βE2), 30 min before Aβ, caused about a 30% reduction of neuronal death. A similar effect was observed when neurons were exposed to 10 nM 17βE2 24 h before challenge with Aβ. The mGlu1 receptor agonist (RS)-3,5-dihydroxyphenylglycine (DHPG;100 µM) reduced Aβ-induced neuronal death by about 25%. The effect produced by DHPG was due to the interaction with mGlu1 receptors expressed in neurons, as, in our conditions, astrocytes do not express this receptor subtype. When mixed neuronal cultures were co-treated with 17βE2 and DHPG, the protective effect against Aβ toxicity was increased. To confirm the specificity of the observed effect, protection exerted by 17βE2 was prevented by the ER antagonist ICI 182,780 (1 µM), but also by a 30 min pre-treatment with the selective mGlu1 receptor antagonist JNJ 16259685 (JNJ; 100 nM) that, when added alone, did not modify neuronal viability. To exclude a possible interaction of ERs with mGlu5 receptor, a similar set of experiments was carried out in the presence of the mGlu5 receptor selective antagonist 2-methyl-6-(phenylethynyl)pyridine hydrochloride (1 µM). Also in these conditions, 17βE2 and DHPG protected against Aβ challenge, an effect reverted by treatment with JNJ. To determine whether a physical interaction between ER and mGlu1 receptor occurs, control and 17ßE2-treated extracts of pure cortical cultures were pulled down with anti-ERα and then blotted against mGlu1 receptor. Exposure for 30 min to 10 nM 17ßE2 increased ERα/mGlu1 receptor co-immunoprecipitation. To investigate at post-receptor level, attention has been focused on the phosphatydilinositol-3-kinase/AKT pathway that is known to be activated in response to both ER and mGlu1 receptor stimulation. The increased phosphorylation of AKT induced by a 30 min exposure to 10 nM 17ßE2 was reduced in the presence of the mGlu1 receptor antagonist JNJ, further supporting an interaction between the two receptors. Accordingly, inhibition of AKT signalling by 10 µM 10-[4'-(N,N-diethylamino)butyl]-2-chlorophenoxazine hydrochloride counteracted the protective effect of 17ßE2, DHPG or both against Aß-induced toxicity. Our results suggest novel mechanism for the neuroprotective effect of estrogen, involving the interaction of ERs, specifically ERα and mGlu1 receptors.

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Transthyretin aggregates affect viability and electrical properties of cardiomyocytes

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Senile systemic amyloidosis (SSA) is a sporadic disease affecting aged people whose main symptom is a severe cardiomyopathy associated with arrhythmias. SSA is characterized by the presence of extracellular amyloid fibrillar aggregates of transthyretin (TTR), a plasma protein carrying the thyroid hormone and the retinol binding protein. The aggregates are deposited in several tissues and, together with their oligomeric precursors, are responsible for tissue functional impairment. To date, liver and heart transplantation are the only medical treatments of SSA; accordingly, a thorough investigation of the molecular basis of cell/tissue functional and viability impairment induced by TTR aggregates is expected to provide knowledge needed to identify new pharmacological targets and to develop novel therapeutic strategies.
We studied the effect on electrophysiology and viability of HL-1 cardiomyocytes of prefibrillar and fibrillar aggregates of TTR supplemented to the culture media. Only the prefibrillar aggregates were able to interact with the cell membrane and were internalized. This resulted in a moderate impairment of cell viability at the lowest aggregate concentration (10 μM). In the same cells exposed to TTR prefibrillar aggregates, the cytosolic calcium content showed a slow, progressive rise over time; it did not reach a steady state level and came back to its basal levels upon TTR removal from bath solution. By the patch-clamp technique we investigated the effect of the enhanced intracellular calcium on the electrical properties of isolated mouse ventricular myocytes. Action potential recordings were performed at increasing rate of stimulation (0.5, 1, and 2 Hz) before and after application of TTR prefibrillar aggregates. The results showed a progressive prolongation of the action potential that was associated with a marked increase of the duration of the plateau phase; eventually, early and delayed afterdepolarizations occurred. These effects were seen at any frequency of stimulation. Altogether, our data indicate the presence of electrical abnormalities in the exposed cells with pro-arrhythmic potential induced by aggregated TTR. On-going investigation will assess the ionic basis of the altered intracellular calcium and clarify the mechanisms of TTR aggregate cytotoxicity particularly at the level of the cell membrane, the endoplasmic reticulum, and the mitochondria. To our knowledge, this is the first mechanistic demonstration of a direct proarrhythmic effect of TTR aggregates in cardiomyocytes, a possible cause of SSA cardiomyopathy.

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Montelukast prevents microparticles-induced inflammatory and functional alteration in human bronchial smooth muscle cells

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Microparticles (MP), also referred to as microvesicles or ectosomes, are membrane fragments shed by virtually all eukaryotic cells upon activation or during apoptosis, which play a significant role in pathophysiological processes, including coagulation and inflammation. Recently, monocyte/macrophage-derived MP were demonstrated to contribute to the innate immune response of different cells, since they activate the release of IL-8 and other cytokines in the airway epithelium and increase PGE_2 production in synovial fibroblasts. The release of inflammatory cytokines and a reduced responsiveness to β_2-adrenoceptors (β_2-AR) stimulation represent features which characterize airway inflammatory diseases such as asthma.
Montelukast (MTK), a potent and selective antagonist of cysteynil leukotriene receptors (CysLTRs), was recently awarded of CysLTRs independent anti-inflammatory activity. The aim of this study was to investigate whether MP derived from monocytes/macrophages stimulate IL-8 release and affect β_2-AR responsiveness in human bronchial smooth muscle cells (hBSMC). Moreover, the influence of a treatment with MTK on MP-induced effects was evaluated. Monocytes/macrophages were isolated from the buffy coats of blood donor by Ficoll gradient centrifugation, followed by overnight culture of the mononuclear cell fraction. Adherent cells were washed and incubated with the calcium ionophore A23187 and the MP-containing supernatant was incubated with human bronchial smooth muscle cells. IL-8 release was measured by ELISA, COX-2 and phosphodiesterase 4D5 (PDE4D5) gene expression were assessed by RT-PCR; besides, the β_2-AR responsiveness was evaluated as increase in intracellular cAMP levels, measured by cAMP enzyme immunoassay kit. MP treatment enhanced IL-8 release in bronchial smooth muscle cells (3-fold increase over basal) and reduced β_2-AR responsiveness by 60% when compared with untreated cells. RT-PCR data evidenced that MP increased both COX-2 and PDE4D5 enzymes expression. MTK (30 μM) for 24 h inhibited significantly MP-stimulated IL-8 production and β_2-AR desensitization. These preliminary findings suggest that MP participate to airway inflammation by promoting cytokine release and altering functional response in hBSMC; finally, MTK appears to inhibit both these effects and therefore has a protective activity against immunological and functional changes.

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Reciprocal potentiation of the antitumoral activity of FK866, an inhibitor of NAD^+ synthesis, and traditional chemotherapeutic agents

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There is a constant need to improve anticancer drugs both to treat tumours with unmet needs and to circumvent tumour resistance. Indeed, these new anticancer drugs should attempt to exploit novel mechanisms of action. Recently, several anticancer agents have been described to induce cell death by manipulating cellular energy stores. In this respect, it has been proposed that interfering with NAD^+ levels might lead to tumour cell death, as these cells have high usage rate of this pyridine nucleotide compared to normal cell. Indeed two compounds, named FK866 and CHS-282 (Hasmann et al. 2003; Hjarnaa et al. 1999), are in phase II of clinical trials (www.clinicaltrials.gov). These compounds are inhibitors of Nampt, the rate limiting enzyme in the NAD^+ salvage pathway. Indeed, contrary to previous thoughts, it has now been demonstrated that NAD^+ can be consumed by a number of enzymes, including PARPs and sirtuins. In this context, we decided to investigate: i) whether neuroblastoma cell lines were sensitive to FK866 and the mode of cell death induced by FK866; ii) the possibility to use Nampt inhibitors as a chemo-sensitizing agent.
In this respect we found that FK866 leads to cytotoxicity in neuroblastoma cell lines and this correlates with a decrease in NAD^+ levels. However apoptosis is not the major cell death pathway engaged. Indeed FK866 leads to macro-autophagy in neuroblastoma cell lines (Billington et al. 2008), that seems to be due to inhibition of mTOR. Surprisingly the co-treatment with 3-MA, an inhibitor of autophagy, is able to partly revert the cell death induced by FK866, suggesting that maybe the induction of autophagy is not the best strategy to kill cancer cells. In fact we found that is better to treat cancer by a multidrug approach combining Nampt inhibitor with traditional chemotherapeutic agents. The co-treatment with FK866, at a concentration that *per se* does not induce cytotoxicity, potentiates cell death induced by cisplatin or etoposide. Indeed, cisplatin and etoposide concentration-response curves are shifted to the left by about 2 orders of magnitude. This reciprocal potentiation appears to be due to a massive NAD^+ depletion in cytosolic and mitochondrial compartments; on the contrary the treatment with FK866 alone leads only to a cytosolic NAD^+ depletion.
Taken together, these data suggest that manipulating NAD^+ levels in different compartments by Nampt inhibitors and traditional anticancer agents could be a better strategy for cancer therapy.

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Differential post-transcriptional regulation of COX-2 expression in human umbilical vein endothelial cells derived from diabetic and healthy women: role of microRNAs

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COX-2 is one of the vasoprotective genes up-regulated by steady laminar shear stress (Topper et al. 1996; Di Francesco et al. 2009) and produces the vasoprotective prostacyclin (Grosser et al. 2006; Di Francesco et al. 2009). Early investigations have shown that diabetes markedly alters prostanoid synthesis in the vasculature (Bagi et al. 2006) but studies on the potential involvement of COX-2 in diabetic vascular complications have given controversial results. The aim of this study was to investigate whether the exposure to a diabetic environment *in vivo* could affect the regulation of endothelial COX-2 expression through post-transcriptional mechanisms. Thus, we compared COX-2 expression in human umbilical vein endothelial cells derived from normal (nHUVEC) and type I diabetic mothers (dHUVEC). Confluent monolayers of cells at passage level 3 were treated with or without IL-1β 5 ng/ml for 6 or 24 h in medium 199/DMEM (50:50) supplemented with ECGF, pen-strep, glutamine, and 5% FCS. The medium was assayed for 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, PGD_2 by radioimmunoassay or ELISA, cell lysates for COX-1, COX-2, microsomal PGES-1 (mPGES-1), prostacyclin synthase (PGIS), and heme oxygenase (HO)-1 by specific Western blot techniques. RNAs were extracted and analysed for COX-2, microRNA(miR)542-3p, and miR16 by real time-PCR. In dHUVEC there was a statistically significant increase in the biosynthesis of 6-keto-$PGF_{1\alpha}$, PGE_2, and PGD_2, but not $PGF_{2\alpha}$, in response to IL-1β vs nHUVEC. The values of prostanoid generation are reported in the table.
Prostanoid levels measured in nHUVEC and dHUVEC after IL-1β (5 ng/ml) for 6 and 24 h (values are reported as ng, mean±SEM, n=5-7; *P<0.05 vs nHUVEC)

	6-keto-$PGF_{1\alpha}$		PGE_2		$PGF_{2\alpha}$		PGD_2	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
nHUVEC	2.8±0.5	13.7±2.1	3.0±0.7	9.2±3.0	22.7±6.9	122.4±29.8	0.6±0.1	1.1±0.3
dHUVEC	9.9±3.6*	37.7±10.5*	5.4±1.0	23.2±5.2*	35.2±14.7	132.1±54.6	0.8±0.3	2.4±0.7*

In both types of HUVEC, prostanoids were generated by COX-2; in fact, pretreatment with 1 μM NS-398 (a selective inhibitor of COX-2) caused an almost complete inhibition of prostanoid biosynthesis. After IL-1β treatment, Western blot analysis showed that COX-1 and PGIS protein levels were comparable in nHUVEC and dHUVEC while mPGES-1 levels were undetectable both in nHUVEC and dHUVEC. The protein levels of COX-2 were significantly (P<0.05) higher in dHUVEC vs nHUVEC at 24 h (COX-2/β-actin optical density 2.30±0.50 vs 0.88±0.12, respectively). mRNA levels of COX-2 in response to IL-1β were significantly (P<0.01) higher in dHUVEC than in nHUVEC (17.98±1.50 vs 5.91±0.60 at 6 h; 9.16±0.60 vs 3.46±0.70 at 24 h, respectively). Experiments of mRNA stability performed in the presence of actynomycin D (0.65 μg/ml), to inhibit transcription, demonstrated that COX-2 mRNA was more stable in dHUVEC than in nHUVEC after IL-1β stimulation; in fact, at 3h there was 30% vs 13% of COX-2 mRNA remaining in dHUVEC and nHUVEC, respectively (P<0.01). We analysed the levels of two different miRNAs involved in the destabilization of COX-2 mRNA through their binding in the 3'UTR: miR542-3p and miR16. In nHUVEC at 24 h with IL-1β, miR542-3p and miR16 levels were significantly (P<0.05) increased vs unstimulated cells (relative miRNA levels: miR542-3p, 2.2±0.8 vs 1.00±0.02; miRNA 16, 1.71±0.26 vs 1.04±0.02, respectively). On the contrary, in dHUVEC stimulated with IL-1β for 24 h, miR542-3p and miR16 levels were lower than in unstimulated cells. Interestingly, miR542-3p and miR16 levels were lower in dHUVEC vs nHUVEC, at 24 h with IL1β (0.53±0.20 and 0.74±0.20, respectively; P<0.05). HO-1 expression was higher (P<0.01) in HUVEC from diabetic than healthy women, in response to IL-1β and NS-398 reduced HO-1 levels both in nHUVEC and dHUVEC. In summary, IL-1β induces COX-2-dependent prostanoids in HUVEC. Prostacyclin is a dominant autocrine prostanoid. HUVEC exposed to a diabetic environment express higher levels of COX-2 through post-transcriptional mechanisms. Loss of IL-1β-dependent inducibility of miRNA-542-3p and miR-16 in dHUVECs is associated with enhanced COX-2 whose expression was associated with higher levels of HO-1. In conclusion, targeting of miRNAs in endothelial cells may represent a new therapeutic strategy to modulate COX-2-dependent prostacyclin.

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Increase of myeloid microvesicles in the cerebrospinal fluid as biomarker of microglia/macrophage activation in neuro-inflammatory disorders

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We have recently shown that the typical danger signal ATP, which accumulates in the brain during inflammation, induces shedding of microvesicles (MVs) from cultured microglia. In addition we have shown that microglia-derived MVs store and release the pro-inflammatory cytokine IL-1β, thus representing an unconventional pathway of protein secretion (Bianco et al. 2005; Bianco et al. 2009). In the present study we aimed at verifying the existence *in vivo* of microglia-derived MVs and at investigating whether MVs contribute to the inflammatory process.
Cerebrospinal fluid (CSF) represents an easily accessible source of material for diagnosis and monitoring of neurological diseases. It is a potential indicator of abnormal CNS states such as inflammation, infection, neurodegenerative processes and tumor growth (Zougman 2008). CSF is the only body fluid in direct contact with the brain and therefore can act as a recipient of shedding products release by neuronal cells. Therefore, to investigate the presence of microglia-derived MVs *in vivo* we analyzed by electron microscopy, Western blotting, and flow cytometry, the CSF collected from healthy rodents. We found that rodent CSF contains MVs of microglial origin, similar in size and content to MVs released *in vitro* from microglia. Of note, flow cytometry analysis of microglia-derived MVs indicated increased levels of MVs in the CSF of mice intracerebrally injected with lentiviral vectors codifying for IFNγ or TNFα, a protocol known to induce dramatic activation of microglia/macrophages. These results represent the proof of principle that the amount of microglial MVs in the CSF reflects the activation state of microglia *in vivo* and thus the extent of the inflammatory condition. In line with these results microglia-derived MVs also increase in rodent CSF in the course of experimental autoimmune encephalomyelitis (EAE), a widely used model for human multiple sclerosis, reflecting disease activity. In both chronic and relapsing EAE, the number of microglia-derived MVs is closely associated to disease course, increasing with EAE severity and decreasing with recovery.
We have previously demonstrated that shedding of MVs is abolished in glial cells established from acid sphingomyelinase (A-SMase) knock out mice (Bianco et al. 2009). To evaluate whether microglia/macrophage MVs actively contribute to neuroinflammation *in vivo*, we induced EAE in A-SMase knock out mice. Our data idicated that A-SMase knock out mice were clinically resistant to the induction of EAE. These findings link microglia activation to the propagation of MVs *in vivo* and identify CSF MVs as novel *in vivo* biomarkers of microglia activation and active contributors of brain inflammation.

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Influence of genetic variants and post transcriptional factors on imatinib transporters as determinants of the pharmacological response

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Imatinib mesylate (IM) is the first example of targeted therapy among tyrosine kinase inhibitors and is widely accepted as gold standard in the treatment of chronic myeloid leukaemia (CML; de Kogel et al. 2007). Despite emerging success in CML therapy, some patients experience suboptimal response or resistance, which highlights the need to find biological predictors of response in order to guide therapy optimization. Over-expression of Bcr-Abl gene, mutation on ATP-binding site, abrogating IM binding, or altered expression of influx (hOCT1, OCTN, OATP) and efflux (ABCB1, ABCG2) transporters, critical determinants of intracellular drug levels, may be responsible of resistance to IM (Kim et al. 2009). The aim of this study was: 1) to investigate the role of genetic variants (SNPs) in genes encoding for IM transporters, as candidate of IM responsiveness; 2) to test the influence of miRNAs on IM response, focusing in particular on efflux transporters. The purpose was to investigate whether miRNAs, as responsible of post-transcriptional regulation, are altered under IM-treatment and could therefore contribute to the mechanisms of IM-resistance (Garofalo et al. 2008). Firstly, a panel of SNPs was genotyped in a subgroup population of 189 patients enrolled in TOPS trial. The associations with cytogenetic response (CgR) and molecular response (MR) was assessed for each SNP. Secondly, an *in vitro* model on chronic myeloid leukemia cell line K-562 IM resistant was established (0.05-10 μM). miRNAs profiles were analyzed using Taqman arrays and *in silico* search was performed for miRNAs deregulated after IM treatment. mRNA expression was quantified using TaqMan realtime PCR and protein expression was analyzed by Western blotting. Treatment outcomes were compared according to the candidate genotypes among Caucasian patients and ABCB1 rs60023214 significantly correlated with complete MR (P=0.005). Concerning SNPs combination in IM uptake transporters the associations with treatment outcomes were statistically significant for both major and complete MR with P=0.005 and P=0.01, respectively. ABCB1 protein was not expressed under any conditions of treatment, differently from ABCG2. The combined analysis of miRNA arrays and *in silico* search revealed two deregulated miRNAs both having ABCG2 as potential target. Under acute IM treatment one miRNA showed up-regulation in a concentration dependent way, whereas the second miRNA was twofold down-regulated under any IM concentration. Under chronic treatment at 0.2 μM IM the first miRNA showed 3.10±2.88 fold down-regulation (P=0.050) and at concentration higher than 1 μM both miRNAs were significantly up-regulated at 3 μM (4.36±2.04 and 2.36±0.60 fold, respectively; both with P=0.05). The exchange of expression pattern of the investigated miRNAs could be functionally confirmed on mRNA and protein level. Experiments of loss and gain of function confirmed the functional influence of the identified miRNAs on ABCG2. The multiple candidate gene approach based on the pharmacogenetics of IM identified single and combination of SNPs that can be proposed as predictor of IM MR. Thus, further study are warranted to validate the role of SNPs on the early identification of CML patients who may not respond optimally to standard IM therapy. The results of the *in vitro* study suggest that IM resistance could be mediated by miRNA-dependent mechanism and miRNA expression could provide a new approach to predict anticancer drug resistance.

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Palmitoylethanolamide exerts anti-inflammatory and neuroprotective effects through PPARα involvement in models of beta-amyloid toxicity

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In the last few years, a growing amount of evidence underlines the crucial role that reactive gliosis plays in the development and progression of neurodegenerative disorders, including Alzheimer's disease (AD). Indeed, the accumulation of the beta amyloid peptide (Aβ) to form senile plaques is considered one of the prominent cause of AD and an important inducer of neuroinflammation. Aβ-activated glial cells mediate an inflammatory response in order to protect the stressed tissue and to restore its physiological condition. Unfortunately, prolonged astrocytic stimulation triggers an abnormal release of pro-inflammatory mediators and cytokines that, in turn, leads to a further recruiting of glial cells amplifying the inflammatory insult and harming neighbouring neurons. This results in an expansion of the neuropathological damage (Skaper 2007; Glass et al. 2010). Based on the evidence that neuroinflammation turns from a defensive role into a detrimental process, the study purpose was to investigate possible anti-inflammatory and neuroprotective properties of palmitoylethanolamide (PEA) in models of Aβ-induced reactive gliosis. Indeed, PEA is an ALIAmide that mimics several endocannabinoid activities with no interaction with cannabinoid receptors (LoVerme et al. 2005). It has been recently reported PEA anti-inflammatory effects in some neuropathological conditions, other than AD (Duncan et al. 2009; LoVerme et al. 2005).
Firstly, it was tested the anti-inflammatory effect of PEA treatment (100 nM) on primary rat astrocytic cultures challenged with Aβ (human fragment 1-42, 1 µg/ml). Results showed a significant reduction of all the inflammatory parameters studied, such as the release of NO, TNFα, and IL1β in culture medium, the expression of both GFAP and S100B, two specific markers of astrocytic activation, as well as the expression of iNOS and COX-2, proteins importantly involved in the inflammatory process. All the data obtained confirm PEA ability to reduce astrocytic activation and to attenuate the inflammatory process in the model studied. Although recent studies have suggested the involvement of peroxisome proliferator-activated receptor alpha (PPARα) in the mechanisms of PEA actions (Duncan et al. 2009; LoVerme et al. 2005), the exact site implicated in the neuroinflammatory effect observed remains still unknown. PPARα belong to the family of nuclear hormone receptors and their physiological ligands are steroids and lipid metabolites. Among other effects, their activation results in a modulation of the inflammatory response (Moraes et al. 2006). Therefore, experiments were repeated challenging Aβ-stimulated astroglial cultures with PEA (100 nM) in the presence or absence of MK886 (3 µM) and GW9662 (9 nM), selective antagonists of PPARα and PPARγ, respectively. Results showed that MK886 was able to revert significantly PEA anti-inflammatory action in all the parameters of inflammation studied, suggesting a PPARα selective involvement in PEA action.
Further investigations were carried out to verify if PEA was able to exert a rebound protective effect on neurons thanks to its capability to attenuate reactive gliosis. *Ex vivo* experiments were performed on rat hippocampal organotypic cultures challenged with Aβ (human fragment 1-42, 1 µg/ml) and PEA (100 nM) in the presence or absence of the two PPARs antagonists. Morphological analyses indicate that Aβ caused a severe damage in the hippocampal regions vs control, whereas PEA was able to counteract this detrimental effect. Moreover, results showed that PEA-treated hippocampal slices displayed serious neuronal damage and loss when PPARα was selectively blocked, indicating its involvement in PEA neuroprotective action.
In conclusion, data from *in vitro* studies reveal that PEA is able to significantly blunt Aβ-induced expression and release of pro-inflammatory molecules and that these effects are PPARα-mediated. Findings from hippocampal organotypic cultures show the neuroprotective properties of PEA and indicate PPARα as the molecular target upon which PEA exerts its neuroprotective effect here reported. These evidence underlines the strictly connection between neuroinflammation and neurodegeneration, supporting the hypothesis that the reduction of reactive gliosis results in a significant attenuation of neuronal degeneration, suggesting a novel bidirectional approach to treat neurological conditions, in which the neuroinflammation plays a pathogenetic role.

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Characterization of the anticancer and antimetastatic properties of ruthenium-based drugs and identification of the signalling pathways involved

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Over the last decade cancer therapy has been moved towards a different direction: targeting DNA is no longer the main strategy. Indeed, novel molecules that interfere selectively with cellular pathways abnormally active in either primary or secondary tumors were developed.
In recent years, ruthenium-based molecules have emerged as promising antitumor and antimetastatic agents with potential uses in platinum-resistant tumors or as alternatives to platinum (Antonarakis et al. 2010). Despite the promising results, the development of these drugs still encounters several “resistances”. One is the difficulty to efficiently screen for antimetastatic ruthenium-based molecules without using too simplistic *in vitro* or ethical-scientific challenged *in vivo* models. The second is to identify precise molecular mechanisms used by ruthenium-based drugs in cancer cells. Finding a solution for these problems are precisely the objectives of my PhD.
The first aim of my project is to investigate the molecular basis of ruthenium-based drugs effectiveness in treating cancer, which seems to be different to other molecules belonging to the same class of chemicals, such as cisplatin. At the moment, our attention is focused on RDC11, a molecule that showed interesting *in vivo* anticancer properties in multiple models (Meng et al. 2009). Based on Affymetrix micro-array analyses, we decided to focus on one specific cellular process particularly relevant for cancer: the pathway of hypoxic stress response mediated by hypoxia-inducible factor 1 (HIF-1). HIF-1 is a transcription factor stabilized in response to hypoxic stress and capable of activating genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism, and invasion (Semenza 2003). Our results indicate that RDC11, unlike cisplatin, is able to decrease HIF-1α expression in both SW480 and HCT116 colon adenocarcinoma cell lines maintained in hypoxic condition to mimic the tumoral environment. In agreement with this evidence, RDC11 modulated in a similar way also the expression of HIF-1β and phospho-S6 ribosomal protein, which are two proteins of HIF-1 pathway. Moreover, RT-qPCR showed that RDC11 treatment decreased the expression of two HIF-1 target genes such as FIH-1 (factor inhibiting HIF-1) and GLUT-1 (involved in glucose metabolism) in the two cells lines under both normoxic and hypoxic conditions. Thus, RDC11 seems to be involved in HIF-1 inhibition and this could explain, at least in part, the ability of ruthenium-based compounds to block tumor development.
The second aim of my project is to assess the antimetastatic potential of ruthenium-based molecules, such as RDC11 and several of its derivatives. To improve the modelization of the complex process of metastases that involve detachment, migration, invasion and re-adhesion of neoplastic cells to a distant site (Chiang et al. 2008), we developed a bioreactor (called plastic mouse). By using interconnected co-culture systems to recreate the environment of either a primary tumor (represented by HT-29 colorectal cancer cells growing on HCEC colon healthy cells) or the target tissue (represented by IHH immortalized hepatic cells), we could follow the migration and invasion of the target tissue by cancer cells *in vitro*. Experiments will be soon in progress to evaluate the antimetastatic behaviour of novel anticancer agents such as RDC11. Clearly, the plastic mouse is a device that can be used to recreate different tumor models. For this reason, we will try also to adapt it to the dissemination of glioblastomas towards healthy nervous tissues.
Altogether, our results give an explanation of the possible molecular mechanisms of action of ruthenium-based drugs and, also thanks to the use of an innovative experimental device, we showed that these compounds can likely target cellular pathways distinct from DNA replication and metabolism.

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