4th PF₂MUC Symposium

Personalized Medicine: Myth or Reality?

Dia da Faculdade de Medicina
Universidade de Coimbra

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Auditorium, Central Unit, Polo III
Health Science Campus

Book of Abstracts
4th PF₂MUC Symposium

Personalized Medicine: Myth or Reality?

Organizing Committee:
Ana Ledo, Inês Violante, Maria Ribeiro, Raquel Santiago

Sponsors:
Foreword

The PostDoc Forum of the Faculty of Medicine (PF₂MUC) has been invited by the Direction of FMUC to organize the scientific session of the Day of Faculty 2013.

The PostDoc Forum is a platform for postdoctoral scientists to network and develop activities related to their interests (www.uc.pt/en/fmuc/pf2muc).

This year’s PF₂MUC symposium will focus on the ethical and medical issues of Personalized Medicine.

Additionally, an important part of the symposium is the “Made in FMUC” rapid fire and poster presentations, a showcase of all the research taking place at FMUC.

We are grateful for the support from the Faculty of Medicine.

We would like to specially thank Prof. Manuela Grazina for the help organizing the program and the evaluators for their contribution.

Finally, we appreciate the sponsorship from Adega Cooperativa do Távora.

On behalf of the PostDoc Forum,

The organizing committee
Ana Ledo, Inês Violante, Maria Ribeiro and Raquel Santiago
Program

14h00  **Opening ceremony**
Prof. Dr. Joaquim Murta (Director FMUC),
Dr. Francisco Ambrósio (Vice-Director FMUC) and
Dr. Ana Ledo (PF2MUC Organizing Committee)

14h10  **How do genes transform the Medical approach to treatments – translating Pharmacogenomics**
Prof. Dr. Manuela Grazina (Laboratory of Biochemical Genetics and FMUC)

14h25  **The personalization of therapy in the context of hepatic dysfunction**
Prof. Dr. Rui Manuel Santos (FMUC)

14h45  **Rapid Fire Oral Presentations I “Made in FMUC”**

15h00  **Poster session and coffee break**

16h00  **Rapid Fire Oral Presentations II “Made in FMUC”**

16h15  **Legal issues and genetic counseling for individualization of Medicine**
Dr. Lina Ramos (Clinical Genetics Department, CHUC)

16h45  **Personalized medicine: challenges and legal frame**
Dr. Marisa Matias (Centre for Social Studies, UC; Member of the European Parliament)

17h15  **Round Table**
Moderator: Prof. Dr. Lino Gonçalves (CHUC, FMUC)
Prof. Dr. Manuela Grazina (Laboratory of Biochemical Genetics and FMUC)
Dr. Lina Ramos (Clinical Genetics Department, CHUC)
Dr. Marisa Matias (Centre for Social Studies, UC; Member of the European Parliament)

18h00  **Awards and Closing ceremony**

18h15  **“Espumante de honra” – Adega Cooperativa do Távora**
Invited Speakers
Manuela Grazina, PhD


Rui Manuel Santos, MD PhD

Rui Manuel Carvalho Marques Santos iniciou a sua passagem pela Universidade de Coimbra em 1971 e é agora docente da Faculdade de Medicina (FMUC). No campo profissional dedicou-se ao estudo das doenças do fígado e em particular da fibrose hepática e dos efeitos do álcool. A qualidade dos cuidados de saúde e a educação médica são áreas que também lhe merecem uma atenção especial. Coordena ainda, em colaboração com o professor Pedro Ferreira da Faculdade de Economia da Universidade de Coimbra (FEUC), o curso pós-graduado de Qualidade e Segurança em Cuidados de Saúde.
Lina Ramos, MD

Presidente da Sociedade Portuguesa de Genética Humana. Licenciada em Medicina pela Faculdade de Medicina da Universidade de Coimbra em 1989. Actualmente desempenha a sua actividade no Serviço de Genética Médica do Centro Hospitalar e Universitário de Coimbra (consulta de genética e aconselhamento genético no Hospital Pediátrico, consulta de aconselhamento genético na Maternidade Bissaya Barreto e observação e colheita de produtos biológicos de fetos após IMG, mortes fetais e de recém-nascidos).

Autora de diversos artigos científicos na área da genética médica.

Marisa Matias, PhD

Lino Gonçalves, MD PhD

Professor Associado com Agregação da Unidade Curricular de Cardiologia da Faculdade de Medicina da Universidade de Coimbra; Chefe de Serviço de Cardiologia, Serviço de Cardiologia, Hospitais da Universidade de Coimbra. Trabalhou durante dois anos (1997-1998) nos National Institutes of Health (Bethesda, USA) onde dirigiu de uma forma autónoma, durante 18 meses, a Secção de Farmacologia e Fisiologia Experimental do Serviço de Cardiologia.

Faculty of Medicine, University of Coimbra is sponsoring two travel awards for the best oral and poster presentations by FMUC affiliated researchers.

**Best poster presentation: 500 €**  
**Best oral “rapid fire” presentation: 750 €**

One hundred and twelve abstracts were submitted, and evaluated by an independent panel.

Six abstracts were selected for a five minutes oral “rapid fire” presentation, and will enter the “Best oral rapid fire” competition.

During the event, an evaluation panel will score Posters and “rapid fire” oral communications. Prizes will be awarded accordingly.

Abstracts shortlisted for oral presentation:

- **Rui Baptista**  
  Micro-RNA 424 modulates the pivotal BMPR2 pathway of pulmonary arterial hypertension through targeting of SMURF1

- **Helena Carvalheiro**  
  The role of CD8+ T cells in rheumatoid arthritis

- **Lídia Costa**  
  Can epigenetic modulation be a new therapeutic approach to treat Diffuse Large B Cell Lymphoma?

- **Maria Madeira**  
  Role of adenosine A2A receptor in the control retinal neuroinflammation

- **Ana Pina Rodrigues**  
  Noise exclusion processing in developmental dyslexia: an eye movement study

- **Vanessa Coelho-Santos**  
  Methamphetamine-induced permeability in brain endothelial cells
Evaluation panel

Ana Bela Sarmento Ribeiro
Ana Cristina Carvalho Rego
Ana I. Duarte
Antero Abrunhosa
Célia Gomes
Cláudia MF Pereira
Cristina Januário
Dan Brudzewsky
Filipa Baptista
Filipe Silva
Flávio Reis
Francisco Oliveira
Henrique Girao
Isabel Prata
Joana Gonçalves
Lorena Petrella
Margarida Carneiro
María Filomena Botelho
Paula Agostinho
Paula Faria
Paulo J. Oliveira
Rodrigo A. Cunha
Rosa Fernandes
Sandra Carvalho Bos
Sandra Morais Cardoso
Tatiana Rosenstock
Teresa Gonçalves
Abstracts

Selected for oral presentation
# RF1

**Micro-RNA 424 modulates the pivotal BMPR2 pathway in pulmonary arterial hypertension through targeting of SMURF1**

R Baptista1,2, C Marques2, G Castro1, P Monteiro1,2, L Gonçalves1,2, P Pereira2, H Girão2

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**Keywords:** Pulmonary hypertension; Pulmonary arterial hypertension; SMURF1; microRNA-424; ubiquitin

Idiopathic pulmonary arterial hypertension (PAH) is a devastating condition defined by sustained elevation of pulmonary vascular resistance due to excessive vascular remodeling and vasoconstriction in the pulmonary arterioles, ultimately leading to right heart failure and death. Rare familiar forms of PAH involve in most cases mutations on the BMPR2 gene that deregulate the BMP signaling pathway. This pathway signals to the nucleus via the receptor-regulated SMADs (R-SMADs). The SMAD ubiquitylation regulatory factor-1 (SMURF1) attenuates these signals by targeting SMAD for ubiquitin-dependent degradation by the proteasome. Therefore, mechanisms involved in the regulation of the levels of SMAD contribute to modulate the BMPR2-mediated signaling. In our study we hypothesize that the microRNA-424, that has been very recently associated with PAH, either through the regulation of the stability of HIF-1α by targeting cullin2 or through the apelin-FGF2 pathway, targets SMURF1. The data observed in this work demonstrates, for the first time, that SMURF1 is a direct target of microRNA-424. Moreover, we demonstrate that miR-424 treatment of HEK293A cells restores BMPR2 signaling upon BMP4 stimulation. This effect is dose-dependent and similar to that induced by SMURF1 depletion by a specific siRNA. Altogether, these results sharply suggest that microRNA-424 constitutes a new regulatory player in the BMPR2 pathway, paving the way to new potential therapeutical approaches developed against PAH.
# RF2

**The role of CD8+ T cells in rheumatoid arthritis**

Carvalheiro H¹, Duarte C³#, Silva-Cardoso S¹#, Souto-Carneiro MM¹,², da Silva JAP²,³

1. Immunology, Center for Neurosciences and Cell Biology, University of Coimbra, Portugal; 2. Faculty of Medicine, University of Coimbra, Portugal; 3. Department of Rheumatology, Centro Hospitalar Universitário de Coimbra, Portugal

# both authors contributed equally to this work

**Keywords:** CD8 T cells; rheumatoid arthritis; autoimmunity.

There is a growing body of data suggesting that CD8+ T cells may be involved in autoimmune diseases, such as multiple sclerosis, encephalomyelitis, diabetes mellitus and vitiligo. These cells were found to be accumulating and undergoing clonal expansion on lesions as well as in the peripheral blood of the affected patients, where they appear to be exposed to their cognate antigen.

The immune system is also known to play the foremost role in the pathogenesis of rheumatoid arthritis. As our team has demonstrated, CD8+ T cells accumulate in the arthritic joints of the K/BxN mouse model for spontaneous chronic arthritis, which produce high amounts of pro-inflammatory cytokines and granzyme B. CD8+ T cells are thus believed to contribute to joint destruction but they are also likely to contribute to the recruitment of other effector cells into the arthritic joints.

These results are essentially paralleled by our findings in human RA. When comparing RA patients’ with healthy controls’ peripheral blood, we found that even though RA patients have a significantly lower percentage of CD8+ T cells in circulation, these have a higher amount of short-term (CD69+) and medium-term (CD25+) activated and effector cells (CD27-CD62L-), while the memory subset (CD27+CD62L+CCR7+) is significantly reduced. We also observe a higher percentage of CD8+ T cells producing pro-inflammatory cytokines, such as IL-6 and TNFα, as well as the lytic enzyme granzyme B, which are consistent with the cytotoxic function of CD8+ T cells. We also observed a higher production of the anti-inflammatory cytokine IL-10, which infers that CD8+ T cells also have an important suppressor activity in this disease.

We found that CD8+ T cells are enriched in the synovial fluid of RA patients. These cells also have a mainly short-term activated (CD69+), effector (CD27-CD62L-CCR7-) and effector memory (CD27+CD62L-) phenotype and express high levels of the homing receptor CXCR4. They also produce high amounts of pro-inflammatory cytokines, in this case IL-6 and TNFα.

The overall role of CD8+ T cells in the whole RA process seems to be the result of a poor regulation and balance of both types of responses triggered by CD8+ suppressor and cytotoxic T cells in the joint. Understanding the dynamics of these responses and the factors regulating them can hold important clues to new approaches to understand and treat this challenging disease.
Can epigenetic modulation be a new therapeutic approach to treat Diffuse Large B Cell Lymphoma?

Costa L(1,2), Domingues C(2), Alves R(2,3,4), Gonçalves AC(2,3,4), Sarmento-Ribeiro AB(2,3,4,5)

1. Medical Student, Faculty of Medicine, University of Coimbra (FMUC), Portugal; 2. Applied Molecular Biology and University Clinic of Hematology, Faculty of Medicine, University of Coimbra (FMUC), Portugal; 3. CIMAGO – Centre of Investigation on Environment, Genetics and Oncobiology, FMUC, Portugal; 4. CNC – Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; 5. CHUC - Hospital and University Centre of Coimbra (CHUC)

Keywords: Diffuse large B cell lymphoma; Epigenetics; Decitabine; Trichostatin A

Diffuse Large B Cell Lymphoma (DLBCL) is the common denomination for a group of different neoplasms that comprise together between 25 to 40% of all non-Hodgkin lymphomas, whose incidence is rising. Classically, it is classified as high-grade lymphoma, being prevailing among men, whose median age is placed between the sixth and seventh decades of life. Current standard chemotherapeutic regimen allows remission in more than half patients. However, about 40% fail to respond or relapse not only to it, but also to rescue chemotherapeutic regimens and radiotherapy. These patients usually hold a poor prognosis, thus bringing an additional motivation for targeted therapy. Epigenetic emerges as a possibility of modulating gene expression, allowing the re-expression of silenced tumor suppressor genes and inhibiting oncogenes.

Here, we studied the potential therapeutic of a hypomethylating agent, decitabine (DAC), and a histone deacetylase inhibitor, trichostatin A (TSA), in monotherapy and in therapeutic association, in a DLBCL cell line (Farage cells).

Farage cells were cultured at a concentration of 0.5x10^6 cells/mL, in RPMI medium supplemented with fetal bovine serum, in the absence or presence of TSA, DAC, and vincristine (VCR), in monotherapy and in double or triple combinations of these drugs. We used a range of concentrations for TSA from 10 nM to 500 nM, for DAC from 1 µM to 25 µM, and VCR at 0.1 nM. After incubation, every 24 hours during a total period of 72 hours, cell proliferation was accessed using resazurin test. Cell death was analyzed after 48 and 72 hours of incubation by flow cytometry using propidium iodide (PI) and annexin-V (AV). Caspase activity was measured with ApoStat Apoptosis Detection Kit. Cellular morphology was analyzed by optical microscopy of cell smears stained with May-Grunwald Giemsa.

Our results show that both TSA and DAC decrease cell proliferation in a time and dose dependent manner. After 72 hours of incubation, TSA, in monotherapy, was more effective than DAC, reducing respectively cell proliferation in approximately 30% (cell viability of 68.5%±7.6% for TSA 50 nM), and nearly 20% (cell proliferation: 83.2%±9.7% for DAC 2.5 µM). The association of these two epigenetic modulators showed a synergistic effect (cell proliferation of 42.7%±11.2%), as well as in combination with VCR (cell proliferation of 32.3%±8.2% for TSA plus VCR and of 36.6%±16.5% for DAC plus VCR). Furthermore, the combination of the three drugs was the best therapeutic regimen tested (cell proliferation of 13.3%±15.3%). These compounds in monotherapy and in therapeutic association induce cell death by apoptosis, which are in agreed with the increase in caspases activity and morphological characteristics.

Our study suggests that epigenetic modulation might be a new therapeutic approach in Diffuse Large B Cell Lymphoma (DLBCL).

This work is supported by Centre of Investigation in Environment Genetics and Oncobiology (CIMAGO).
Role of adenosine A2A receptor modulation in the control retinal neuroinflammation

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Keywords: Microglia; retina; adenosine; neuroinflammation

Activation of microglial cells is a characteristic of the neuroinflammatory response associated with neurodegenerative diseases. This activation can lead to an exacerbated expression of pro-inflammatory mediators that can be potent inducers of cell death.

Increasing evidence has demonstrated that blocking adenosine A2A receptor (A2AR) may prevent neurodegeneration by modulating the release of noxious factors by activated microglia. Little is known about the role of microglial cells in the control of the neuroinflammatory responses in the retina. The aim of this work was to evaluate the effect of A2AR blockade in the control of retinal neuroinflammation.

Purified retinal microglial cell cultures and cultured retinal explants were pretreated with A2AR antagonist, and challenged with LPS to mimic an inflammatory stimulus.

Expression of A2AR increased in cultures of retinal microglial cells and retinal explants after exposure to LPS. A2AR blockade inhibited LPS-induced ROS increase and decreased the percentage of microglial cells incorporating fluorescent latex beads, as compared to LPS condition, indicating that blockade of A2AR prevents the increase in phagocytic activity. In cultured retinal explants, blockade of A2AR modulated the LPS-induced morphological changes in microglial cells. A2AR blockade decreased the LPS-induced expression of TNF, IL-1β and iNOS and reduced the release of the pro-inflammatory cytokines IL-1β and TNF.

Our results indicate that A2AR may have a role in the inflammatory responses in the retina, opening the possibility of the use of A2AR antagonists in retinal degenerative diseases involving inflammation.

Noise exclusion processing in developmental dyslexia: an eye movement study

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Keywords: Developmental Dyslexia; eye movements; noise exclusion

Developmental dyslexia is a highly prevalent neurodevelopmental disorder. It is characterised by a reading and writing deficit despite a normal level of intelligence. It has been shown that dyslexics make different eye movements during reading when compared to normal readers. It has also been suggested that dyslexics have deficits in excluding surrounding noise. In this study, we aim to understand the influence of noise in reading deficits by measuring eye movements during word reading under different levels of noise in dyslexic and normal children.

Two groups of children with (N=27) and without dyslexia (N=49) were included. Regular, irregular and pseudowords were randomly presented in the centre of the screen in three background noise conditions: no noise (clear white background), symbol noise (abstract symbols) and white noise (random bright and dark dots). Subjects had to discriminate between words and pseudowords by pressing a button. Eye movements were measured using a SMI remote eye tracker.

A Repeated Measures analysis indicated a main effect for group for the accuracy rate (p<0.05), which was lower for dyslexics. Concerning eye movements, a Repeated Measures ANOVA showed a main group effect (p<0.01) regarding the number of fixations and fixation duration. As expected, overall, dyslexic children did more and longer fixations than controls. An interaction effect for Group x Noise (p<0.05) was also found for the fixation duration. The number and duration of fixations in the control group were modulated by both noise conditions (symbols and white noise). On the other hand, in the dyslexic group, the duration of fixations and the number of fixations was only influenced by the white noise.

The different eye movements patterns found in dyslexics suggest a different noise exclusion processing in these children, which may play a role in the reading deficits that characterize this condition.
Methamphetamine-induced permeability in brain endothelial cells

Vanessa Coelho-Santos\textsuperscript{1,2}, Carlos Fontes-Ribeiro\textsuperscript{1,2}, Ana Paula Silva\textsuperscript{1,2}

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Keywords: Adhesion molecules, blood-brain barrier, endothelial cells, methamphetamine and tight junctions.

Methamphetamine (METH) is a powerful psychostimulant drug of abuse that causes severe alterations in the central nervous system (CNS). Recently, some studies have suggested that the damage associated with METH might result from its ability to compromise the blood-brain barrier (BBB) function. However, the mechanisms underlying the effect of this drug of abuse on BBB are still poorly understood.

The aim of this work was to clarify how METH affects BBB properties. For that, bEnd.3 cell line and rat brain microvascular endothelial cells (RBMECs) were used, since barrier-conferring cellular equivalent of the BBB are the endothelial cells (ECs) of the brain capillary network. Our results demonstrate that low concentrations of METH (0.001-3 mM for 24h exposure) did not interfere with ECs cell viability in both cultures. Additionally, the analysis of METH-induced alterations in both transendothelial electric resistance (TEER) and paracellular permeability were performed in RBMEC. We concluded that 1 or 50 μM METH decreased the TEER and increased the permeability to sodium fluorescein (376 Da). Moreover, in an attempt to identify the key players in METH-induced ECs permeability we further investigated potential alterations in tight junction (TJ) proteins. Occludin and ZO-1 protein levels were downregulated by 1 or 50 μM METH (24h exposure).

We have also previously shown that METH triggers a neuroinflammatory response. So, we further investigated possible alterations in intercellular adhesion molecule (ICAM-1) levels, since it is expressed constitutively at low levels on ECs but can be significantly increased in the presence of cytokines and reactive oxygen species. Indeed, a significant increase of ICAM-1 protein levels was triggered by 1 or 50 μM METH.

In summary, our results show that METH did not induce EC death but negatively interferes with barrier properties, which can be in part explained by a decrease in TJ expression.

(This work was supported by Project PTDC/SAU-FCF/098685/2008, and a research fellowship (BI) from the same project Strategic Project PEst-C/SAU/UI3282/2011-COMPETE and FEDER funds)
Abstracts

Aging
Proteasomal dysfunction in aged retinal pigment epithelium contributes to Age-related Macular Degeneration progression.

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Keywords: Age-related macular degeneration; Retinal pigment epithelium; Proteasome; Drusen

Age-related macular degeneration (AMD), the leading cause of blindness in elderly people of developed countries, is a complex and multifactorial disease. One of its cardinal features is the accumulation of extracellular deposits called drusen, between the retinal pigmented epithelium (RPE) and Bruch’s membrane. Beginning early in life, and continuing throughout lifespan, cells of the RPE gradually accumulate molecular debris, as a consequence of their high phagocytic activity. These residual materials are remnants not only from phagocytic activity but also from incomplete degradation of abnormal/damaged proteins. It has been reported that activity of the ubiquitin-proteasome pathway (UPP) in the RPE decrease upon aging. Previous studies demonstrated that expression of ubiquitin in which lysine 6 is replaced by tryptophan (K6W-Ub) impairs the function of UPP. The main objective of this work was to investigate the hypothesis that chronic impairment of UPP in RPE contributes to some of the cardinal features of AMD, including drusens. To address this question ARPE-19 cells were infected with empty vector, K6W Ubiquitin mutant and Wild Type Ubiquitin. The drusen formation and accumulation was evaluated in a basal matrix by confocal microscopy, using specific drusen markers such as APOE, while exosome release was obtained by isolation through ultracentrifugation and determined by western blot (CD63 and Tsg101) and flow cytometry. The results obtained showed that expression of K6W ubiquitin mutant leads to an impairment of proteasome activity and an increased amount of exosomes release and drusen accumulation.
Abstracts

Cancer
Hypoxia induced alterations on 18F-FDG metabolism - An in vitro study in three different colorectal carcinoma cell lines

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Keywords: colon cancer, hypoxia, 18F-FDG, p53

Introduction: Recent clinical data indicate that tumor hypoxia negatively affects the treatment outcome of radiotherapy and chemotherapy in various cancers, emphasizing the need for noninvasive detection of tumor hypoxia. The application of 18F-FDG PET imaging in oncology is based on the upregulation of glucose transporters and glycolytic enzymes, and tumor hyperglycolysis. In this context, the main objective of this study is to determine the pattern of uptake of 18F-FDG in three colorectal carcinoma cell lines in normoxia and hypoxia conditions and correlate these results with the expression of GLUT-1, -3 and p53.

Methods: Studies were performed in colorectal carcinoma cell lines (WiDr, C2BBe1 and LS1034). Uptake studies with 18F-FDG were carried out. After incubation in a cell suspension of 2×10^6 cells/ml (25µCi/ml), samples were collected, radioactivity of pellets and supernatants was measured and percentage of uptake calculated. Experiments were conducted in normoxia and hypoxia environment. GLUT-1, -3, as well as p53 expression was assessed by flow cytometry and western blot, respectively.

Results: p53 protein quantification revealed that C2BBe1 cell line does not express p53 while WiDr and LS1034 do. Related to 18F-FDG uptake, LS1034 and WiDr cell lines increased the uptake in hypoxic conditions in contrast with C2BBe1. Concerning GLUT-1 and -3 expression we observed that hypoxia (2 and 48 hours) induced an increase of these glucose transporters.

Conclusions: With these results, we can conclude that in solid tumors, as colorectal cancer, the uptake of current radiotracers is influenced by tumor microenvironment. Besides that, characteristics like GLUTs, the main glucose transporters, can be responsible for these results. However, the genetic background also reveals be a key role in cells uptake.
Targeted therapy in gastric carcinoma: Automated system for HER2 IHC and FISH

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Keywords: HER2, gastric carcinoma, IHC, FISH

Introduction
Anti-HER2 transtuzumab benefit in Her2 overexpression and amplification in 10-20% of gastric carcinoma (GC) implies testing. Two scoring systems for immunohistochemistry-IHC and FISH runs in the same fully automated staining system were compared to validate quickness and economical sparing.

Material and Methods
Sections of 49 biopsies of GC were either fully automatically stained with monoclonal antibody-based OracleHER2 Bond IHC kit and registered after the breast scoring for GC results and tested for amplification with dual-colour LSI HER2/CEP17 Dual Probe in the automated system BOND-MAX™.

Results
HER2 FISH amplification and overexpression - six cases (13%) IHC 3+ also positive for FISH; of 2 cases (4%) IHC 2+, one was FISH positive (HER2/Chr17= 2.8) and the other was FISH negative (HER2/Chr17= 1.0) due to polysomy of chromosome 17; 2 cases IHC 1+, were FISH negative (HER2/Chr17= 1.6 and 1.5).

Discussion
The automated BOND-MAX™ staining system for IHC and FISH demonstrated overall good quality in observed slides. Our study identified 6 cases with aberrant in situ hybridization polysomy of chromosome 17, negative for HER2 FISH amplification. Described heterogeneous tumor FISH patterns of chromosome 17 aneusomy appear as Her-2/CEP17 low ratio and were classified as negative. The applied methodology revealed to be economical with less manual discrepancy for routine in Pathology.
ABC Expression as Additional Marker to monitoring response in CML - A Preliminary Study

Alves R (1,2,4), Gonçalves AC (1,2,4), Couceiro P (3), Rodrigues-Santos P(3), Freitas-Tavares P(5) and Sarmento-Ribeiro AB (1,2,4,5)

1- Applied Molecular Biology and Hematology University Clinic, Faculty of Medicine, University of Coimbra, Portugal; 2- CIMAGO – Center of Investigation on Environment Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Portugal; 3- Immunology Institute, Faculty of Medicine, University of Coimbra, Portugal; 4 – Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal; 5 – Clinical Hematology Service, Centro Hospitalar Universitário de Coimbra (CHUC), Portugal.

Keywords: Chronic myeloid leukemia, ABC transporters, Molecular Response

Chronic myeloid leukemia (CML) is a mieloproliferative disorder characterized by the presence of the BCR-ABL gene fusion, which encodes an oncoprotein with a deregulated tyrosine kinase activity. This oncoprotein became the main therapeutic target of the disease with the introduction of specific tyrosine kinase inhibitors (TKIs). Additionally, the fusion gene expression level is used as a monitoring tool to determine the molecular response of each patient. However, several patients show incomplete or loss of response and even in some cases resistance to the TKI therapy. Leukemia stems cells, as normal stem cells, could present ABC transporters to protect them-self against therapeutic agents which could contribute to therapeutic failure. One of the most described ABC transporters is PgP and Imatinib and other TKIs are substrates for these efflux transporters. The precise link between ABC transports and response to therapy in CML is not fully understood.

The aims of this work were to evaluate the expression levels of ABC transporters, namely PgP and BCRP, in CML patients and to correlate them with the BCR-ABL levels.

We examined in CD34+ cells obtained from peripheral blood of 46 patients with CML, the expression levels of PgP and BCRP by flow cytometry using monoclonal antibodies. The BCR-ABL quantification was determined according to the international guidelines for CML management and at the time point of the study 26 patients (56,5%) present a positive quantification of BCR-ABL while the remain 20 patients (43,5%) were BCR-ABL negative. The median patient age at diagnosis was approximately 48 years (24–88), gender M/F=24/22, and 95% of the patients are under treatment with TKIs.

Our preliminary results show that patients with no detectable BCR-ABL mRNA (negative) present a higher percentage of CD34+ cells than patients positive for this fusion gene. Furthermore, 80% of these negative patients (n=16) expressed PgP and/or BCRP in cell membrane and 56,2% of these patients would present a positive quantification of BCR-ABL levels in the first 6 months and 75% at the end of one year.

These results suggest that the presence of ABC transporters in CD34+ cells of patients without detectable levels of BCR-ABL could constitute an earlier marker of loss of response and may be an additional tool for monitoring response in CML patients. However, further analysis should be performed, namely an increased number of patients, to confirm our results and the clinical relevance.

This work was supported by Center of Investigation on Environment Genetics and Oncobiology (CIMAGO) and FCT grant SFRH/BD/51994/2012.
Nordihydroguaiaretic acid as a new therapeutic approach in cancer

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Keywords: Nordihydroguaiaretic acid; Hepatocellular carcinoma.

Cancer is the second leading cause of death worldwide, making this disease responsible for 12.5% of deaths. Consequently, the pharmaceutical industry has focused on developing anticancer drugs more potent and effective. In fact, the history of anti-cancer therapy is closely related to natural products, which represent over 60% of medicines used in this pathology. Since the twentieth century, clinical trials were developed in order to identify, develop and approve new active compounds derived from plants with anti-cancer activity, like nordihydroguaiaretic acid (NDGA).

Nordihydroguaiaretic acid (NDGA) is a phenolic compound produced by Larreatridentata, a creosote bush of Mexico and American southwest, known for its anti-oxidant and anti-inflammatory properties. Recently, it has also shown that this compound inhibits cell growth and induces apoptosis in different types of cancer, in both cell culture and animal models. However, the mechanism involved in its anti-tumorigenic effects are not clear.

The aim of this study was to test the cytotoxic and antiproliferative effects of NGDA in different Hepatocellular carcinoma (HCC) cell lines with different ethiology as well as study the mechanisms involved in the process of cell death

For this purpose, 3 HCC cell lines, Huh-7, Hepg2 and Hep3b, were incubated in the absence and presence of the natural compound NGDA. The antiproliferative effect was assessed by the Alamar Blue assay and cell death was analyzed by flow cytometry using Annexin V and Propidium iodide. We also evaluated oxidative stress levels in these cells upon incubation with natural’s compounds by analyzing the intracellular ROS levels by flow cytometry using specific fluorescent probes, DHE and DCF-DHA, respectively for superoxide anion and peroxides.

Our results showed that NGDA have antiproliferative and cytotoxic effects in a dose, cell and time dependent manner, inducing cell death in all cell lines, preferentially by apoptosis. This effect may be mediated by the increase in intracellular ROS levels.

Our results suggest that NGDA may constitute a new potential therapeutic approach in HCC treatment owing to its pro-oxidant properties when used in high concentrations.
18F-FDG an alternative to 99mTc-MIBI in the study of Multidrug Resistance in Hepatocellular Carcinoma

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Keywords: Multidrug resistance, Hepatocellular Carcinoma, 18F-FDG, 99mTc-MIBI

Background: Hepatocellular carcinoma (HCC) is known to be resistant to chemotherapy, which is due in part to overexpression of multidrug resistance proteins (MDR). A method to evaluate the function of these proteins involves the measure of radiolabeled substrate 99mTc-MIBI uptake. Studies have demonstrated that 18F-FDG uptake is associated with MDR proteins expression in HCC. Other studies have demonstrated that 18F-FDG uptake by HCC is associated with p53 expression since tumors with lower expression or mutated expression of this protein has a higher uptake of this tracer. This study aims evaluate the uptake and retention of 18F-FDG and 99mTc-MIBI in three human HCC cell lines and to correlate them with the expression of three MDR proteins and with p53 expression.

Methods: Cell lines used were HepG2 (wp53), HuH7 (mp53) and Hep3B2.1-7 (p53 under-expressed). Uptake and retention studies with 18F-FDG or 99mTc-MIBI were performed. Cells grown in low glucose medium (5mM) and in high glucose medium (25mM) in order to verify the influence of glucose on 18F-FDG and 99mTc-MIBI uptake and retention. Pgp, MRP1 and LRP proteins expression were determined by flow cytometry. To evaluate MDR modulation, retention studies were performed in the presence of verapamil (Pgp inhibitor) prior to incubation with 18F-FDG or 99mTc-MIBI.

Results: For all cell lines used, 18F-FDG and 99mTc-MIBI uptake and retention were higher when cells grown on low glucose medium. For both media formulations Hep3B2.1-7 cell line has higher uptake and retention of 18F-FDG and 99mTc-MIBI. HepG2 cell line has a lower uptake and retention and a higher expression of MRP1. Through modulation studies with cells incubation with verapamil, a considerable increase of 18F-FDG and 99mTc-MIBI retention in all cell lines were obtained.

Conclusions: It is concluded that medium glucose concentration influences the uptake and retention of both radiopharmaceuticals. There is an inverse relationship between MRP1 expressions and uptake and retention of 99mTc-MIBI and 18F-FDG. Through modulation studies it was found that Pgp has an active role on MDR in HCC. Uptake and retention profiles for the two radiopharmaceuticals are similar, showing that the 18F-FDG can be used to study the MDR proteins function in HCC cells, being an alternative to 99mTc-MIBI. We also conclude that p53 expression influences 18F-FDG uptake once Hep3B2.1-7 and HuH7 cell lines have higher uptake than HepG2.
PET Scan SUVs and Mitochondrial DNA copy number correlate with histological sub-typing in Bronchial-Pulmonary Carcinoma

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Keywords: PET, mitochondrial DNA copy number, Bronchial-Pulmonary Carcinoma

Introduction
Bronchial-Pulmonary Carcinoma remains with poor survival as near 70% of cases are diagnosed in non-surgical stages. Epidermoid Carcinomas comprising common histological criteria have better prognosis than the other types while Adenocarcinomas still comprehend a number of heterogeneous subtypes, complicated with polymorphic carcinomas that have obscured biology. PET SUV Max 18F-Fluordesoxiglucose (FDG) and tumoural mitochondrial DNA (mtDNA) copy number were used to validate their value as clinical laboratorial parameters to correlate with histological and clinical diagnosis.

Material and Methods
Tumoral samples of surgical specimens of 8 Epidermoid Carcinomas, 5 TTF1+ Adenocarcinomas, 4 Pleomorphic Carcinomas and 4 Large Cell Neuroendocrine Carcinomas combined with Adenocarcinomas (2) and with Epidermoid Carcinomas (2) were submitted to DNA extraction by the Maxwell® 16 FFPE Tissue LEV DNA Purification Kit, mtDNA copy number was determined by real-time PCR assay and correlated with PET Max-18-FDG.

Results
Significant difference (p=0.006) was found between Epidermoid Carcinomas and TTF1+ Adenocarcinomas 18-FDG uptake. Pleomorphic Carcinomas and Combined LCNEC showed 18-FDG uptake in between the former histological types; mtDNA copy number and SUVmax were correlated using linear regression and statistical significant direct proportionality was found (p=0.0059, r²=0.2485).

Discussion
These results reinforce the role of tumoural classification for “personalized” treatment based in biological characteristics as SUVmax and mtDNA copy number correlated. These characteristics should be considered when biopsy tissue is the unique representation of advanced pulmonary carcinomas. The recent PET scans interpretation comment of G. Weiss and R. Korn (JTO Dec 2012) sustain the biological approach here presented.
**Vismodegib (GDC-0449): a new therapeutic approach in hematological neoplasias treatment**

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**Keywords:** Hematological neoplasias; Hedgehog pathway; Vismodegib (GDC-0449); targeted therapy

The Hedgehog (Hh) signaling pathway regulates tissue polarity, patterning and stem cell maintenance during embryogenesis. In adults the Hh pathway is mainly quiescent, except in tissue maintenance and repair. However, recently, the hyperactivation of this pathway, by either mutation or deregulation, has been linked to tumorigenesis in a wide variety of tissues, namely in hematopoietic tissue. On this way, development of agents that target critical steps in the Hh pathway will may constitute a new successfully therapeutic strategy in hematological malignancies. Take into account, the aim of this work is to evaluate the therapeutic efficacy of the hedgehog inhibitor, vismodegib, in hematological neoplasias.

For this purpose we maintained in culture CEM (Acute Lymphoblastic Leukemia cell line), HL-60 (Promyelocytic Leukemia cell line), K-562 (Chronic Myeloid Leukemia/erythroleukemia cell line) and FARAGE (Diffuse Large B-Cell Lymphoma cell line) cells and tested the effect of different concentrations of vismodegib (GDC-0449) during 24 to 72 hours. Cell viability was assessed by the trypan blue and alamar blue assays and cell death by Optical Microscopy (May-Grunwald staining) and flow cytometry (FC) using the Annexin V/Propidium Iodide (PI) double staining. Some of the mechanisms involved as BAX/BCL-2, caspases, p53 and cyclin D1 expression levels were also evaluated by FC using specific monoclonal antibodies. Cell cycle arrest and mitochondrial membrane potential were also assessed by FC using PI solution and JC-1 probe, respectively. Our results showed that vismodegib induces antiproliferative and cytotoxic effects in a dose, time, administration schedule and cell type dependent manner. The half maximal inhibitory concentration (IC50) at 48h is 75 μM, 100 μM and 200 μM, respectively for HL-60, CEM and K-562 cell lines. On the other hand, in FARAGE cells the IC50 was not reached. This compound induces cell death mainly by apoptosis in agreement with the observed increase in caspases levels and BAX/BCL-2 ratio and the decrease of mitochondrial membrane potential. Moreover, vismodegib induces antiproliferative effects through cell cycle arrest in G2/M phase which may be correlated with the decrease in p53 and cyclin D1 levels.

In conclusion, our study suggests that vismodegib (GDC-0449) may constitute a new potential targeted therapeutic approach in hematological neoplasias, namely in acute leukemias.

This work is supported by Center of Investigation in Environment Genetics and Oncobiology (CIMAGO).
**Butyrate in the prevention of colorectal cancer: a study in three colorectal cancer cell lines**

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**Keywords:** colon cancer; butyrate; cancer prevention; cytotoxic effect

**Background:** An important diet based on high levels of dietary fiber is related with a lower risk for developing colon cancer. Butyrate, a short-chain fatty acid, is a main end-product of intestinal microbial fermentation of mainly dietary fiber, being an important energy source for colonocytes and, it plays an important role in maintenance of the colon homeostasis. It was also reported that butyrate may be able to be a chemopreventive agent. The aim of this study is to evaluate the effect of butyrate on three colorectal cancer cell lines.

**Methods:** WiDr and C2BBe1 (colorectal adenocarcinoma) cells were cultured in DMEM whereas LS1034 (cecal carcinoma, multidrug resistant cell line) cells were cultured in RPMI. Cells were incubated with different sodium butyrate concentrations (1-50 mM). In order to determinate the IC50 (half maximal inhibitory concentration) at 24, 48, 72 and 96 hours, cell proliferation was evaluated by the MTT assay. Flow cytometry was also performed to study the butyrate effect on cell viability and types of death, apoptosis (evaluating the bax/bcl2 ratio) and expression of reduced glutathione (GSH).

**Results:** The treatment with increasing doses of butyrate at 24h, showed a slight decrease of cell proliferation. However, it was observed that when cells are subjected to butyrate for a longer time (48h, 72h and 96h) cell proliferation decrease with low IC50 values. These results are similar in all cell lines, being LS1034 cells the most sensitive to butyrate at longer incubation times. Regarding cell viability, preliminary results showed that as the butyrate concentration increases, cell viability decreases in all cell lines. When C2BBe1 and LS1034 cells are incubated with higher butyrate concentrations, there is an increase in bax/bcl2 ratio. There is also a slight increase in GSH expression, comparing to control.

**Conclusions:** Our study suggests that butyrate has an anti-proliferative and cytotoxic effect on the three CRC cell lines despite of the different genetic background and organ localization. In order to obtain constant and ideal concentrations of butyrate in the colon, a rich diet in certain fibers is needed, and maybe, by hypothesis, it may contribute for preventing the inflammation and cancer.
Natural killer adoptive cell transfer targeting bladder cancer stem cells Preliminary results for fine-tuning strategy

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Keywords: Cancer stem cells; Natural killer cells; Immunotherapy; Bladder cancer

Bladder cancer (BC) is characterized by an aggressive phenotype with high propensity for recurrence and/or metastasis, probably related with the presence of cancer stem cells (CSC). Natural Killer (NK) cells are lymphocytes able to kill a wide range of cancer cells due to its powerful cytolytic activity, being considered suitable candidates for adoptive immunotherapy. We aim to explore the role of BC-CSC in the susceptibility to NK cell mediated-based immunotherapy. Two human BC cell lines (HT-1376 and UM-UC3) were assayed for their susceptibility to NK cells-induced lysis, using the CD107a-based degranulation assay and the TO-PRO-3 killing assay. The presence of CSC was analyzed using the sphere-forming assay. Cells’ chemosensitivity cisplatin (CIS) and methotrexate (MTX) was determined using the MTT-colorimetric assay. BC cells were also characterized according to NK cell receptor ligands by FACS analysis.

A subset of CSCs was identified in the HT-1376 cell line using the sphere-forming assay. BC cells phenotypic characterization revealed the expression of MICA/B, ULBP1, CD48 and CD58, all ligands for activating NK cell receptors.

Surface expression of CD107a in IL-2 activated NK cells significantly increased (p<0.05) when exposed to UM-UC3 cells (19.25±4.87%), comparing to HT-1376 cells (5.0±2.41%). After 48h of IL-2 stimulation, NK cells significantly increased the capacity of both BC cells lysis, with improved killing in the UM-UC3 cell line.

MTT results showed that HT-1376 cells are more resistant to CIS (5.30±0.98μM) and MTX (0.31±0.06μM; p<0.05) than the UM-UC3 cells (CIS:IC50=3.40±0.61μM; MTX: IC50=0.02±0.01μM).
The sphere-forming HT-1376 cells are more immune and chemoresistant than the UM-UC3 cells. Both BC cell lines are susceptible to NK cell-mediated cytotoxicity, however the UM-UC3 presents higher killing rates and triggers increased NK cell degranulation.
#P11

**mTOR inhibitor in oral cancer – the Influence of Insulin in toxicity induced by Everolimus.**

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**Keywords:** Oral Cancer, PI3K/Akt/mTOR pathway, mTOR inhibitor, Insulin

Oral cancer (OC) is a subtype of head and neck cancer (HNC) that arises from the oral cavity, summing about 40% of the total. The current curative treatment modalities are usually surgery and radiation, with chemotherapy added to decrease the possibility of metastasis. On the other hand, some studies have shown a higher prevalence of cancer in diabetic patients, namely oral cancer.

PI3K/Akt/mTOR is a very important pathway, involved in the regulation of many cellular processes including apoptosis, cell proliferation and cell metabolism and its deregulation is considered pivotal for oral cancer and a potential candidate for therapeutic targeting. Everolimus inhibits cell signaling through the PI3K/Akt/mTOR pathway and has been shown to reduce cell proliferation and angiogenesis. It is also known that insulin can indirectly activate PI3K/Akt/mTOR pathway increasing resistance to anti-cancer drugs, even the mechanism is not well understood yet.

With this work, we intend to study the influence of insulin in toxicity induced by the mTOR inhibitor Everolimus, in oral cancer cell lines.

For this purpose, two oral cancer cells lines, HSC-3 (metastatic) and BICR-10 (in situ) were used. They were incubated with insulin (100uM) and Everolimus in different concentrations, in monotherapy and in association. Cellular toxicity was evaluated by the Alamar Blue assay. The cell death was analyzed by flow cytometry using Annexin V/Propidium iodide assay.

Our results showed that the mTOR inhibitor, Everolimus, had an antiproliferative and cytotoxic effects in monotherapy in a dose, time and cell-dependent manner, inducing cell death preferentially by apoptosis. On the other hand, insulin induces an increase in HSC-3 cells proliferation. Furthermore, in cells pre-treated with insulin we observed a decrease of toxicity mediated by Everolimus, indicating that insulin could influence the sensitivity of oral cancer cells to this mTOR inhibitor.

Our work suggests that insulin can have an important role in chemotherapy resistance in oral cancer which can affect therapeutic protocols in diabetic patients with oral cancer.

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Wnt/β-catenin signaling in Osteosarcoma Cancer Stem Cells

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Keywords: Osteosarcoma, cancer stem cells, Wnt/β-catenin

Osteosarcoma (OS) is a malignant bone tumour afflicting mainly young patients. Although survival has improved substantially since the introduction of multimodal chemotherapy, still 40% of the patients die because of therapy resistant metastasizing tumour cells. Recent studies pointed out for human mesenchymal stem cells as the cell-of-origin of osteosarcoma, and the disease is envisaged as resulting from defective stem cell differentiation. Hence, osteosarcoma appears to be an adequate candidate to fit into the cancer stem cell (CSCs) model, but little is known about the specific mechanisms governing osteosarcoma CSCs’ self-renewal. We therefore aimed to explore the role of Wnt/β-catenin signalling pathway in osteosarcoma CSCs and if its inactivation could sensitize CSCs towards conventional chemotherapy.

Using a well-established sphere assay model, we isolated putative CSCs from osteosarcoma cell lines. CSCs expressed pluripotency-related genes (SOX2, KLF4) and had increased activity of the stem-cell marker Aldefluor®, in comparison with respective parental cells. Importantly, spheres, but not parental cells, contained a fraction of cells with nuclear β-catenin-positivity, which is a hallmark of activated Wnt/β-catenin signalling, as determined by immunohistochemistry. Gene expression in CSC enriched spheres revealed increased constitutive mRNA levels of AXIN2, a specific target gene of activated Wnt/β-catenin signalling, and decreased levels of the secreted Wnt antagonist DKK1 as compared to parental cells.

CSCs were highly resistant to doxorubicin, cisplatin and methotrexate, the first-line chemotherapy applied to osteosarcoma patients, having IC50 values ≥2-fold higher than their parental cells. Pharmacological inactivation of Wnt signalling using IWR-1 (a tankyrase inhibitor) elicited a significant cytotoxic effect on CSCs from spheres, reducing their viability after 48h of drug exposure. Combination of IWR-1 with low doses of doxorubicin, cisplatin or methotrexate diminished cell viability during the same time-period.

Conversely, pharmacological stimulation of Wnt signalling in parental cells using a GSK-3β inhibitor antagonised the sensitivity of cells towards doxorubicin, cisplatin and methotrexate, after 48h of drug incubation, as determined using similar drug combination experiments.

In conclusion, Wnt/β-catenin appears to be specifically activated in the sub-population of CSCs in osteosarcoma. Our results suggest that selectively targeting the Wnt/β-catenin pathway can eliminate CSCs, while current chemotherapeutic agents can only eradicate bulk tumour mass. Combining conventional chemotherapy with Wnt inhibition in the treatment of osteosarcoma can simultaneously contribute to reduce the doses of chemotherapy and eradicate the CSC sub-population, which is thought to be involved not only in cancer progression, but also in recurrence.
#P13

**CAN POLYMORPHISMS IN OXIDATIVE STRESS RELATED GENES BE A RISK FACTOR FOR CML?**

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**Keywords:** CML, Polymorphism, Oxidative stress

Oxidative stress (OS) is recognized to be a evident feature in cancer development and progression. Indirect evidences suggest a role for oxidative stress (OS) in Chronic Myelogenous Leukemia (CML) etiology and pathogenesis. OS, resulting from an imbalance between Reactive Oxygen Species (ROS) production and antioxidant defenses, contributes to cell damage, apoptosis and ineffective hematopoiesis. The antioxidant enzymes superoxide dismutases (SOD) and catalase (CAT) are important components of cell defense against OS, and polymorphisms in the genes may contribute to differences in susceptibility of individuals to oxidative damage since it can lead to reduced protection against OS.

In the present study we set to investigate the influence of polymorphisms of oxidative stress related genes, namely SOD1 (A251G), SOD2 (Ala16Val), COX2 (G-765C), CAT (C-262T) and NADPH oxidase p22 phox (C242T), as a risk factor for CML development.

Our study population consisted of 48 patients diagnosed with CML and the same number of healthy controls. Diagnosis was set according to international criteria. The genetic polymorphisms of SOD1 (A251G), SOD2 (Ala16Val), COX2 (G-765C), CAT (C-262T) and NADPH oxidase p22 phox (C242T) were assessed by RFLP-PCR. The patient group median age was 51 years (18-86), gender M/F=27/21. The strength of association between polymorphisms and CML risk was assessed by odds ratio (OR) with the corresponding 95% confidence interval (CI95%) and Kaplan-Meier survival analysis will be assessed to investigate the prognostic importance of these polymorphisms.

Our preliminary results show a higher wild type allelic frequency of SOD1 (94%), COX2 (80%), CAT (82%) and NADPH oxidase p22 phox (67%) polymorphism and a higher variant allelic frequency SOD2 (52%) in CML patients, compared to controls. In these patients the predominant genotype was AA (88%), CG (52%), GG (67%), CC (73%) and CT (46%), respectively for SOD1, SOD2, COX2, CAT and NADPH oxidase p22 phox. Besides that, individuals with CC genotype of NADPH oxidase p22 phox and with TT genotype of CAT have an increase risk for CML about 2,3636-fold (CI95% 1,0346-5,3997; p=0,032) and 12,368-fold (CI95% 1,5151-100,9687; p=0,0076), respectively. These preliminary results show that CAT (C-262T) and NADPH oxidase p22 phox (C242T) genetic polymorphisms might be related with CML development and may be a novel genetic markers for CML susceptibility.

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May sodium butyrate interfere with 18F-FDG uptake in colon cancer?

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Keywords: Colon cancer; Butyrate; 18F-FDG uptake; Warburg Effect

Background: Butyrate is a short chain fatty acid (SCFA) and it's produced by decomposition of dietary fiber by intestine’s bacteria, being the main energy source of colonocytes. It is related with colon cancer mostly because of its capacity to inhibit histone deacetylases (HDAC), and inducing apoptosis and differentiation in contrast to normal cells. Some studies suggest that the Warburg effect may explain why the cancer cells use preferentially glucose rather than other energy sources, inducing butyrate accumulation in the tumor cells. Other studies also suggest that butyrate can be used by tumor cells as energy source when glucose levels are reduced. The aim of this study is to evaluate if butyrate interferes with uptake of the radiolabeled glucose analogue (18F-FDG) and the increased glycolysis in colorectal cancer cells.

Methods: WiDr and C2BBe1 cell lines were cultured in DMEM with low glucose content (5mM). To perform the uptake studies, cells were incubated with or without butyrate, 3mM for WiDr cells and 15mM for C2BBe1 cells (values chosen taking into account the respective IC50) during 1 and 4 hours, before the incubation with 18F-FDG (25µCi/ml). At 5, 30, 60, 90, and 120 minutes, duplicate samples of 200µl of cell suspension were collected for eppendorfs with iced phosphate buffer solution (PBS). The samples were centrifuged at 10000rpm for 60 seconds, separating the pellet from the supernatant. In order to calculate the 18F-FDG uptake percentage, radioactivity of both fractions was measured in a well-type gamma counter, in counts per minute (CPM).

Results: 18F-FDG uptake is greater in WiDr cells than in C2BBe1 cells, being the uptake at 120 minutes of 6.91%±0.15% and 5.05%±0.15%, respectively. In both cell lines, we observed that incubation with butyrate decreases the 18F-FDG uptake. This difference was more pronounced in WiDr cell line however, in C2BBe1 cells there seems to be a trend for a decrease in tracer uptake with increasing exposure time to butyrate.

Conclusions: Our study suggests that butyrate can reduce the 18F-FDG uptake and may interfere with the Warburg effect which influences the aggressiveness of the tumor. This also suggests that butyrate can act in cancer cells in an advanced phase of development, and could contribute to the understanding of the importance of our diet in advanced tumor stages.
Chemotherapy for adenocarcinomas with neuroendocrine differentiation: a study in two pancreatic cancer cell lines with different somatostatin receptors expression

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Keywords: Pancreatic cancer; adenocarcinoma; neuroendocrine differentiation; chemotherapy; somatostatin receptors

Background: Pancreatic cancer, the fourth leading cause of cancer worldwide, is very resistant to surgery, chemotherapy and radiotherapy. Due to pancreatic cancer resistance to chemotherapy, it’s important to study the single effects of chemotherapy in this type of cancer in order to improve its effects when allied with other treatment strategies.

Material and methods: All studies were performed in two human pancreatic duct adenocarcinoma cell lines with different somatostatin receptors (SR) expression: MIA PaCa-2 (SR++) and Panc-1 (SR+). Cells were incubated with different concentrations of 5-FU or docetaxel for different times (24, 48, 72 or 96h) and cell proliferation was evaluated through MTT assay. Through obtained dose-response curves, half maximal inhibitory concentration (IC50) for 5-FU and docetaxel was achieved. Cells were incubated with drugs IC50 and after 72h, cell viability/death was analysed by flow cytometry using annexin V and propidium iodide.

Results: IC50 was determined for each drug at each time in both cell lines. After 5-FU IC50 incubation, MIA PaCa-2 and Panc-1 cell death reached 47.7% and 23.17%, respectively. Cell death in both cell lines submitted to 5-FU treatment occurred mainly by apoptosis and necrosis. Docetaxel IC50 induced 62.4% of cell death (mainly by apoptosis) in MIA PaCa-2, whereas Panc-1 cell death (33.5%) occurs either by apoptosis and necrosis.

Discussion: 5-FU and docetaxel inhibit cellular proliferation in a dose dependent-manner. Both cell lines are more sensible to docetaxel than to 5-FU. Docetaxel induces a higher percentage of cell death when compared to 5-FU. Docetaxel must be considered as a serious candidate for the treatment of adenocarcinomas with neuroendocrine differentiation.
Methylation pattern in hematological malignancies – A Comparative study between bone marrow and peripheral blood

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DNA methylation status was one of the earliest discovered epigenetic regulators of gene expression and aberrant methylation of gene promoter region is responsible for inappropriate gene silencing, mainly tumor suppressor genes, and it has been associated with cancer initiation and progression. There are several types of specimens from which DNA methylation pattern can be measured and evaluated. Blood-based specimens may be a potential source of noninvasive cancer biomarkers. Blood leukocytes from patients with solid tumors, namely colorectal carcinoma, exhibit complex and distinct cancer-associated DNA methylation patterns, which might be seen as epigenetic biomarkers with significant clinical potential. However, peripheral blood cell methylation profiles are largely unknown in hematopoietic cancers. Our aim was to compare DNA methylation status of four tumor suppressor genes (p15, p16, p53 and DAPK) in Myelodysplastic Syndrome (MDS) and Monoclonal Gammopathy (MG): Multiple Myeloma (MM) and Monoclonal Gammopathy of Uncertain Significance (MGUS), at diagnosis, in bone marrow (BM) aspirate and in peripheral blood (PB).

For this purpose we have examined the methylation pattern of p15, p16, p53 and DAPK genes, in genomic DNA obtained from BM and PB samples collected at diagnosis from 140 patients (MGUS=42; MM=57; MDS=41). Samples were collected after informed consent obtained in accordance with the Helsinki Declaration. Genomic DNA was isolated by standard protocols and modified by sodium bisulphite. The MS-PCR for p15, p16, p53 and DAPK promoter genes was performed using two sets of primers, one for methylated DNA and other for unmethylated DNA.

Overall, 70% and 42% of BM samples and 56% and 39% of PB, respectively from MDS and MG, presented at least one methylated gene. Moreover, we observed discrepant results between BM and PB in 24 of 140 samples (17,1%) for p15 gene, in 20 of 140 samples (14,3%) for DAPK, in 11 of 140 samples (7,9%) for p16 and in 3 of 140 samples (2,1%) for p53 gene. Besides that, discrepant results between BM and PB were highest in MDS patients (p15: 29%, DAPK: 17,1%, p16: 9,8%; p53: 0%) than MGUS (p15: 8,5%, DAPK: 12,7%, p16: 8,5%; p53:4 %) or MM patients (p15: 15%, DAPK: 13,4%, p16: 5,7%; p53: 2%). In some cases discrepancies were also bidirectional, with cases presenting demethylated PB and methylated BM aspirate, and the others presenting methylated PB and demethylated BM.

Our results show that aberrant methylation seems to be a common event in hematological malignancy patients. Moreover, we observed a correlation between gene methylation patterns between peripheral blood and bone marrow aspirate. Although DNA methylation patterns measured in peripheral blood may have great potential as informative biomarkers of cancer risk and prognosis, large systematic and prospective studies will be needed.

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Might Photodynamic Therapy be a solution for Retinoblastoma?

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Keywords: Retinoblastoma, Photodynamic therapy, Photosensitizers

Background: Retinoblastoma (RB) is the most common intraocular disease in children under five years old. The main treatments for RB are radio and chemotherapy; however these treatments could lead to secondary neoplasias due to their mutagenic effects. Photodynamic therapy (PDT) which is a non-mutagenic treatment already used in macular degeneration might be a promising approach to RB. Our previous work comprises the development of several photosensitizers (PS) with good photochemical properties and, more important, excellent cytotoxic effect. The aim of this work was to evaluate the cytotoxic activity of a group of PS previously developed by us: a bromated hydroxyphenyl porphyrin, BBr₂HPP, a bromated hydroxyphenyl chlorine, BBr₂HPC and a glucose acetylated compound, BBr₂HPPGlu-OAC, in vitro in a human retinoblastoma cell line.

Methods: The Y79 human cell line was cultured in RPMI medium supplemented with 15% FBS. Cells were incubated with several concentrations of the PS and irradiated 24 hours later with a total energy of 10J. The half maximal inhibitory concentration (IC₅₀) was calculated 24 and 48 hours after irradiation by colorimetric tests MTT and alamar blue (AB). Flow cytometry was performed to evaluate cell viability, alterations in cell cycle, production of reactive oxygen species and expression of reduced glutathione (GSH).

Results: The preliminary results obtained with MTT and AB assays show that all the PS are cytotoxic at very low concentrations. Dose-response curves obtained through the MTT assay allowed to estimate the IC₅₀s being about 29nM for BBr₂HPP, 20nM for BBr₂HPC and 17nM for BBr₂HPPGlu-OAC. The flow cytometry analysis showed that all photosensitizers promoted a decrease of cell viability with increase in initial apoptosis and late apoptosis/necrosis of cell populations. Evaluation of intracellular production of ROS showed an imbalance of species like peroxides and superoxide anion, being associated a decrease in the expression of GSH. Cell cycle analysis showed an arrested in G2/M phase.

Conclusions: The PS evaluated showed good photodynamic properties. These compounds were able to induce cell death in the retinoblastoma cells with concentrations in the order of nanomolar. These photosensitizers have shown great potential in vitro and in vivo and preclinical studies will be developed soon.
Effect of hydroxyl radical on PC-3 metastatic prostate cancer cell line

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Keywords: Prostate Cancer, Hydroxyl Radical, Oxidative Stress, Hydrogen Peroxide, Iron Chloride.

Prostate cancer is the most commonly diagnosed malignancy in males. Oxidative stress which reflects the imbalance between reactive oxygen species (ROS) and the antioxidant system has been associated to prostate cancer development and progression. ROS are potential inducers of biomolecules oxidative damage. Furthermore, oxidative damage may cause genetic mutations which accumulation contributes to prostate cancer progression. Conventional therapy of prostate cancer, namely chemotherapy, radiotherapy and hormonal treatment are ineffective in the advanced disease due to the development of cancer cells resistance. Some authors explain radiotherapy adaptation, in part, due to the antioxidant system response. Previous results show that an increase of antioxidant defences, in prostate cancer metastasis, induces resistance to an increase of ROS, such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), avoiding hydroxyl radical (\textbullet\text{OH}) formation which is harmful to cells. However, prostate cancer cells resistance against \textbullet\text{OH} is not well established.

In this context, we intend to evaluate if oxidative stress induction, by exposing prostate cancer cells to \textbullet\text{OH}, may represent a new therapeutic approach and/or adjuvant therapy in prostate cancer. Therefore this study was performed in the PC-3 metastatic cancer cell line. Cells were treated with H\textsubscript{2}O\textsubscript{2} and iron (III) chloride (FeCl\textsubscript{3}), alone and in combination, in order to produce \textbullet\text{OH}, via Fenton and Habber-Weiss reaction.

Results show that \textbullet\text{OH} radical, obtained by H\textsubscript{2}O\textsubscript{2} and FeCl\textsubscript{3} combination, induced an inhibitory effect on cancer cells proliferation, higher than the isolated compounds. This inhibition was accomplished by cell cycle arrest during GO/G1 phase, cell death, decrease in peroxides production, which may results from its conversation to \textbullet\text{OH} radical. Likewise, we observed an increase of superoxide anion (O\textsuperscript{2-}) and lipid peroxidation. Moreover, we evaluated the antioxidant defenses response to the increase of \textbullet\text{OH}. We found no modifications on glutathione reductase (Gl-Red) activity and a significant increase of glutathione (GSH) in the cells treated with the compounds alone, but not in the cells treated with the combined compounds reflecting the increase of oxidative stress.

In conclusion, this study suggests that \textbullet\text{OH} obtained by H\textsubscript{2}O\textsubscript{2} and FeCl\textsubscript{3} combination is more effective that H\textsubscript{2}O\textsubscript{2} in the induction of oxidative stress and consequently cell death which may represents a new therapeutic approach in prostate cancer.

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**Development of a new lipid-based nanosystem for specific and efficient gene delivery**

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**Keywords:** Hepatocellular Carcinoma, Asialoglicoprotein Receptor, Gene Therapy, Cationic Liposomes, Lactosyl-PE

Hepatocellular carcinoma (HCC) is considered the sixth type of cancer with the highest prevalence worldwide, corresponding to the third cause of death related to cancer diseases. The current treatment options have many limitations and reduced success rates. In this regard, advances in gene therapy have proven promising results for the treatment of this pathology. However, the success of this therapy depends on the efficient and specific delivery of genetic material into target cells. In this context, the main goal of this study was to develop a new formulation of cationic liposomes, containing the lipid Lactosyl-PE targeted to the asialoglycoprotein receptor (ASGP-R), a membrane protein specifically expressed in hepatocytes and overexpressed in HCC cells, for efficient and specific delivery of genetic material into HCC cells.

Transfection studies with HepG2 cells, showed that the presence of 15% of Lactosyl-PE in the formulation of cationic liposomes induces a strong potentiation of the biological activity of these complexes, not only in terms of transgene expression but also in terms of percentage of transfected cells. In the presence of galactose, which competes with the Lactosyl-PE for the binding to the ASGP receptor, it was observed a reduction in the biological activity levels, showing that the potentiation of biological activity induced by the presence of lactosyl-PE could be due to its specific interaction with ASGP-Rs. In addition, it was found that the presence of Lactosyl-PE into lipoplexes promotes an increase in their cell binding and uptake. Regarding the physicochemical properties of lipoplexes, the presence of Lactosyl-PE resulted in a significant increased in the DNA protection and a substantial decrease in the mean diameter and zeta potential of lipoplexes, conferring them better physicochemical properties.

Overall, our results suggest that the potentiation of the biological activity induced by the presence of lactosil-PE could be due to its specific binding to the ASGP-R overexpressed in HCC, showing that lipoplexes composed by EPOPC:Chol:lactosil-PE (15%)/DNA, in the charge ratio (+/-) 2/1, could constitute a new gene delivery system for application of new therapeutic strategies in HCC.
Hepatocellular carcinoma treatment with amniotic membrane proteins: effects on DNA

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Keywords: Hepatocellular carcinoma; Amniotic membrane; DNA

Background: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy with an increasing incidence worldwide. Overall survival at five years in patients with HCC is between 2% and 10%, being this partially due to the resistance of this cancer to conventional therapies. Human amniotic membrane (hAM) appears to have potential in the treatment of liver diseases, such as cirrhosis or liver fibrosis, which has aroused the researchers’ interest in the last decade. Due to its attractive characteristics, hAM has also recently drawn attention as an upcoming therapy in cancer. However, no studies have focused on the application of hAM in liver malignant disease.

Material and methods: hAM was obtained from healthy women, washed with phosphate buffered solution and subjected to mechanical actions in order to extract proteins, which were quantified using Nanodrop®. To study the effect of hAM proteins extract (hAMPE) in HCC, studies were performed in three cell lines: HuH7 (mp53), HepG2 (wp53) and Hep3B2.1-7 (p53 under-expressed). Cells were incubated with 1µg/µL of hAMPE during 72h. After this period, crystal violet and comet assays were performed to assess DNA synthesis and damage, respectively. In order to analyze early apoptosis related DNA fragments (20-300kb), we used PEG/Hoechst fragmentation assay.

Results: DNA synthesis decreased 75% in Hep3B2.1-7, 66% in HepG2 and 41% in HuH7 cell lines after treatment with hAMPE. Through comet assay can be seen that hAMPE treated HepG2 cells tail moment (product of tail length and fraction of total DNA in tail) increased 64 times relatively to untreated cells. In Hep3B2.1-7 cell line, tail moment increased 3 times when compared to control. There is no difference between hAMPE treated and untreated HuH7 cells tail moment. Regarding PEG/Hoechst fragmentation assay, HepG2 and HuH7 cell lines has a fluorescence ratio between the treated and untreated cells equal to 1.29±0.22 and 1.12±0.18 (MED±SD), respectively. On the other hand, Hep3B2.1-7 (0.84±0.06) has less early apoptosis related DNA fragments.

Conclusion: The treatment of HCC cell lines with hAMPE decreases the DNA synthesis and increases the DNA damage, suggesting that hAMPE may have a promising role in the HCC therapy.
Targeting signaling pathways as a new therapeutic approach in hepatocellular carcinoma: an in vitro study

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Keywords: Hepatocellular carcinoma; Molecular targeted therapy; mTOR inhibitor; Farnesyltransferase inhibitor; Proteasome inhibitor

Introduction
Hepatocellular carcinoma (HCC) is a highly prevalent and lethal neoplasia. Despite its significance, there are only limited therapeutic options, many with negligible clinical benefit. These poor results are related with diagnosis at advanced stages, being rarely amenable to radiotherapy, and with the highly resistance to currently available chemotherapeutic agents. In fact, advances in the understanding of tumor biology open new paths for HCC prevention and treatment through the development of targeted therapies. The design of drugs that regulate cancer-related pathways, such as inhibitors of proliferation or activators of apoptosis, are essential to the opening of new horizons in the HCC treatment.

The aim of this study was to evaluate the therapeutic potential of mTOR (Everolimus), farnesyltransferase (L-744,832) and proteasome inhibitors (MG-262) as new targeted therapies in HCC cell lines in monotherapy and in combination with conventional chemotherapy.

Materials and methods
For this purpose, two HCC cell line, the HepG2 and HUH-7 cells, were cultured in absence or presence of increasing concentration of Everolimus, L-744,832 and MG-262. The cytotoxic effect was assessed by the Alamar Blue assay and the mechanisms of cell death by optic microscopy (after May-Grünwald-Giemsa staining) and flow cytometry (Annexin V/ Propidium Iodide assay). The molecular mechanisms involved in drug cytotoxicity, namely the expression of ubiquitin conjugates, laminin A/C, cyclin D1 and proteins related to cell death (BAX and BCL2), were analysed by flow cytometry using monoclonal antibodies labeled with fluorescent probes. Cell cycle analysis was also performed by flow cytometry (IP/RNase).

Results
Our results showed that mTOR, farnesyltransferase and proteasome inhibitors had antiproliferative and cytotoxic effects in monotherapy in a dose and time dependent manner, inducing cell death preferentially by apoptosis. Furthermore, combination of Everolimus, L-744,832 and MG-262 in lower doses than the IC50, with conventional chemotherapeutic drugs, demonstrated a synergistic cytotoxic effect allowing to reduce the toxicity levels and side effects, which are critical to improve patients survival and quality of life.

Conclusions
Our study suggests that mTOR, farnesyltransferase and proteasome inhibitors may constitute a new therapeutic approach in HCC, either in monotherapy or in association with conventional chemotherapy.
Hepatocellular carcinoma: Interest of microvascular invasion as a factor with impact in prognosis after surgical treatment

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Keywords: Liver; Hepatocellular Carcinoma; Liver Resection

BACKGROUND: Hepatocellular carcinoma (HCC) is the third cause of cancer-related death, and the incidence is growing worldwide. Survival without surgical treatment does not exceed, in most series, 10 months. The option between Resection (HR) and Transplantation (HT), the only options with curative potential, remains controversial. We pretend to evaluate the results of HR of HCC in our department and the interest of microvascular invasion as a factor with impact in prognosis after surgical treatment.

METHODS: 95 patients with medium age of 64±11.7 years (34-85) underwent HR for HCC in our department. 85% were male, 69% had chronic hepatic disease and 90% were classified as Child-Pugh A. Medium size of lesions was 66±48 mm (17-280), 75 were solitaire (79%) and 39% of patients were within the Milan Criteria. Five patients underwent hepatic arterial chemoembolization and ten to portal vein embolization before surgery. Forty-three patients underwent major liver resection and 52 a minor. Prognostic factors were evaluated using univariate and multivariate analyses. Differences were considered to be significant when p≤0.05.

RESULTS: Per-operative mortality (3 months) was 5.5% and morbidity 43.1%. Thirteen patients underwent new surgical treatment due to recurrence, HT in 4 and HR in 9. Overall survival was 58% (5 years) and 36.4% (10 years); disease free survival was 42.3% (5 years) and 26.4% (10 years). In patients with tumours without microvascular invasion overall survival was respectively at 5 and 10 years 75.9% and 56.4%.

CONCLUSION: HR of HCC can be performed with acceptable morbi-mortality, increasing significantly survival in these patients; particularly in those with tumours without microvascular invasion, allowing a better selection of patients that can be treated with HR.
Hepatic Resection of Single and Small Hepatocellular Carcinoma: An Actual Option?

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Keywords: Liver; Hepatocellular Carcinoma; Liver Resection

BACKGROUND: advances in clinical, biological and imagiological studies of patients with chronic hepatic disease allowed an early detection of single and small hepatocellular carcinomas (sshCC). The option between Resection (HR) and Transplantation (HT), the only options with curative potential, remains controversial. The aim of this study is to evaluate the results of HR of the sshCC in our department

METHODS: 95 patients underwent HR for HCC in our department. Twenty-two of them presented a single lesion ≤3 cm (sshCC). The medium age was 62±12,6 years (35-85), 86% were male, all had chronic hepatic disease (81% presented cirrhosis) and 91% were classified as Child-Pugh A. Medium size of lesions was 26±4 mm (17-30). Nineteen patients underwent minor liver resection and 3 a major. Prognostic factors were evaluated using univariate and multivariate analyses;

RESULTS: Per-operative mortality (3 months) was 0% and morbidity 27,3%. Four patients underwent new surgical treatment due to recurrence, HT in 1 and HR in 3. Pathology revealed microvascular invasion in 6 patients. Overall survival was 78% (5 years) and 68,2% (10 years); disease free survival was 72% (5 years) and 57,4% (10 years).

CONCLUSION: HR of sshCC can be performed with acceptable morbi-mortality, presenting overall and disease-free survivals similars to those seen in most series of HT. Our results support that HR is a good alternative to HT in sshCC treatment, and can allow to reduce the number of patients in waiting list for HT.
Clinical implications of early hepatocellular regeneration after portal embolization

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Keywords: Liver regeneration; Two stage hepatectomy; Colo-rectal liver metastases

BACKGROUND: Studies with radioisotopic methods performed by us suggest that hepatic regeneration (HR) in men is fast, occurring a normalization of liver function, 5 days after hepatectomy. This evidence has a high clinical impact, because it makes possible to start adjuvant chemotherapy of surgical treatment earlier and to perform the 2nd stage of an iterative hepatectomy (IH) or an hepatectomy after portal embolization in a short period.

CLINICAL CASE: 70 year-old male underwent a segmental resection of the transverse cólon due to a well-differentiated adenocarcinoma (T3N2M0). Eighten months after surgery 12 bilateral hepatic metástases were diagnosed and patient started neoadjuvant chemotherapy. A good biological [CEA reduction (295,7 to 12,5 ng/dl) and CA19.9 reduction (4552,7 to 131,6 U/ml)] and imagiological (decrease in size of the metastases on CT) response was seen. Nine metastasectomies were performed in the left lobe of the liver (3 in segment II, 4 in III and 2 in IVb) with ligation and alcoholization of the right branch of the portal vein. Six days after surgery CT revealed right lobe atrophy and left lobe hypertrophy. Patient was discharged on 7th postoperative day. Sixteen day after surgery a right IH allowed to resect the remaining metástases. Pathology revealed tumoral necrosis (5% to 10%). Fifteen days after patient started adjuvant chemotherapy.

CONCLUSION: This case demonstrates the clinical significance of translational studies, such as those performed by us, that make possible to optimize therapeutic strategies.
Combined effect of chemotherapy and radiation therapy (Rx) in cell lines of lung cancer

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Lung cancer (LC) is one of the most common malignancies accounting for a large number of deaths in the world, representing 13% of cases and 18% of deaths in 2008. The LC includes small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Being the NSCLC the most common, accounting for about 80% of all cancers. Treatment options are determined by the type and stage of the tumor, the most common chemotherapy and/or radiation therapy.

The aim of this study was to evaluate the effect of radiation (Rx) alone and/or in combination with anticancer drugs, cisplatin, carboplatin and etoposide in cell lines of SCLC and NSCLC.

For this purpose we used two NSCLC cell lines H1299 and A549 and a cell line of SCLC the H69. Cells were grown in appropriate medium supplemented with 5% FBS. The cells were maintained in an atmosphere of 5% CO2 at 37 °C the medium was renewed every 48 to 72 hours.

The cell suspensions of H69, H1299 and A549 were placed at a density of 0.5 × 10⁶ cells/ml and irradiated at different radiation doses (0.5, 15, and 30 Gy) in an accelerator in a single dose. Cells were incubated with different concentrations of cisplatin, carboplatin, and etoposide. Cell proliferation and viability was determined by testing Alamar Blue and Trypan Blue after 24h, 48h, 72h and 96h of treatment. The evaluation of the cell cycle and the type of cell death was performed by flow cytometry 48 hours after irradiation.

The results show that irradiation induced reduction of cell proliferation in all cell lines in a dose dependent manner, with the most effective doses of 15Gy and 30Gy (as expected). In addition, cells treated with the lowest dose of radiation respond in a similar manner to control cells, with inhibition of proliferation and subsequent recovery. In addition to the antiproliferative effect, a decrease in cell viability was observed in a dose and cell line dependent manner, consequently, with an increase of cellular death, mainly by initial apoptosis. In fact, the cytotoxic effect was more evident in A549 and H69 cells than in H1299 cells, suggesting that this line is less sensitive to the effects of RX.

Moreover, when cells were treated with radiation in combination with cytostatic, a cytotoxic effect was observed, dependent on cell line and combination regimen. Thus, in cells which used radiation and cisplatin we observed a synergistic effect on cell lines H69 and A549, while in cell line H1299 this effect was not clear when compared with therapy alone. In cells that we used the radiation combined with etoposide no synergistic effect was observed, relatively to the effect observed with monotherapy.

This study highlights the importance of selecting the appropriate therapeutic schemes to cell type in order to maximize the cytotoxic effect with less secondary toxicity.
DNA Methylation profile in Hepatocellular Carcinoma – A preliminary study

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Keywords: Hepatocellular carcinoma, Epigenetics, Methylation profile

Hepatocellular carcinoma (HCC) is one of the most common primary malignancies of the liver in adults. The tumor usually presents in an advanced stage, when surgical resection is non-curative. Besides the already known genetic mutations found on HCC cells, it has recently been accepted that epigenetic gene expression modifications may play a pivotal role on hepatocarcinogenesis, causing liver tumors molecular heterogeneity. These mechanisms involve CpG islands methylation and histone deacetylation, altering the expression levels of several genes, namely tumor suppressor genes and proto-oncogenes. Aberrant expression of these genes can be associated with tumor progression and etiological risk factors (HBV or HCV infection and alcohol consumption) and also correlated with survival after cancer therapy.

To further understand the role of epigenetics in hepatocarcinogenesis, we have investigated, in a pilot study, the methylation status of some cell cycle and apoptosis regulators related genes in HCC patients.

The study was first performed on DNA from paired fresh frozen tumor, adjacent normal tissues and blood samples obtained from 15 patients (9 HCC, 3 Hepatic Adenomas, 1 fibrotic nodule, 2 cholangiocarcinoma). We analysed the promoter methylation status of cell cycle and apoptosis regulators related genes (GSTP-1, PTEN and DAPK) using the methylation-specific polymerase chain reaction.

Our results show differential methylated patterns in the different blood samples as well as in the tumor and adjacent normal tissue with different etiology. Only in HCC samples we observe a methylated gene profile. Furthermore, the co-existence in some cases of methylated with unmethylated DNA, suggested that both genetic and epigenetic (CpG methylation) mechanisms may act in concert to inactivate gene expression in HCC. However these results need confirmation through an increase in data population.

This preliminary study reinforces the theory that epigenetic modifications may be involved in hepatocarcinogenesis and a better understanding of the global deregulation of methylation status and how they correlate with disease progression will aid in the design of strategies for earlier detection and better therapeutic decisions.

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Characterization of leukocyte infiltration in gastric cancer biopsies and its prognostic impact


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Keywords: Gastric cancer; antitumoural immune response; leukocyte infiltration

Introduction
According to the World Health Organization, gastric cancer represent the fourth most frequent carcinoma, with about 990 000 new cases/year, and the second most fatal, with a total number of 738 000 deaths annually. The immune system plays an important role in controlling the tumour growth, but the poor prognosis of patients with gastric cancer suggests that antitumoural immune response cannot halt the progression of the tumour.

Materials and Methods
Eighty two patients with gastric carcinoma and 33 controls were studied. The leukocyte infiltration, namely neutrophils, monocytes, NK cells, B lymphocytes and T lymphocytes (CD4+, CD8+ and γδ) was assessed by flow cytometry on tumour and normal gastric biopsies using specific monoclonal antibodies.

Results
The increase in the frequency of tumour-infiltrating neutrophils (TIN) correlates with intestinal-type carcinomas, with III-IV TNM stages and with worse overall survival. Whereas the increasing in the frequency of tumour-infiltrating lymphocytes (TIL) is associated with intestinal-type tumours, with I-II TNM stages and with better overall survival. Among the TIL, higher levels of CD4+ T lymphocytes are related with diffuse-type, with I-II TNM stages and with better overall survival; the increase in the percentage of CD8 + and γδ T lymphocytes is correlated with intestinal-type carcinomas, with less advanced TNM stages and with better overall survival. Regarding tumour-infiltrating NK cells, the higher numbers were found in diffuse tumours, in I-II TNM stages and in patients with better overall survival.

Conclusion
Our results suggest that TIN are associated with worse prognosis, whereas NK cells and T cells appear to be involved in the antitumoral immune response and related to better prognosis in gastric cancer patients.
Role of Metformin as co-adjuvant in anticancer therapy targeting Osteosarcoma Cancer Stem Cells

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Keywords: Cancer Stem Cells; Co-adjuvant; Doxorubicin; Metformin; Osteosarcoma.

Osteosarcoma (OS) is the most common malignant primary bone tumor that appears in childhood and adolescence. It was recently demonstrated that OS possesses a small population with stem-like features (CSCs), which are responsible for the heterogeneity and regenerative ability of tumor cells and are refractory to conventional therapies (chemotherapy and radiotherapy). Metformin (METF) is one of the most prescribed drugs to treat type II diabetes and in the past decade METF gained special attention in cancer treatment because of its anticancer properties. In this study we explored the potential role of METF as an adjuvant of doxorubicin (DOX) to target CSCs from OS, exploring the effects and signalling pathways underlying the anticancer properties of metformin on OS CSCs. Both human OS cell lines MNNG/HOS and MG-63 contain sphere-forming cell subsets with stem-like properties expressing Oct4 and Nanog pluripotency markers, which are relatively more resistant to DOX than their differentiated counterparts. METF reduced the proliferation rate and viability of both cell types but was preferentially cytotoxic to CSCs relative to parental cells in a dose-dependent manner, and decreased the sphere-forming and self-renewal ability of both CSCs populations. Moreover, METF potentiate the cytotoxic effects of DOX in both cell populations, although the chemosensitizing effect has been more pronounced against CSCs. METF stimulates FDG uptake in parental differentiated cells but not in CSCs. Exposure to METF induced dose-dependent increase in AMPK activation, with a more pronounced effect in CSCs. METF demonstrated a preferential cytotoxicity against CSCs relatively to corresponding parental cells and decreased the sphere-forming and self-renewal of CSCs. METF induce activation of AMPK and potentiates the cytotoxic effects of DOX mainly in CSCs. Collectively our results suggest that METF combined with DOX may be an effective treatment strategy for targeting CSCs in OS.
Cyclodextrins as phthalocyanines-delivery systems to target and kill cancer cells

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Keywords: cyclodextrins; cancer; photosensitizers; reactive oxygen species

Phthalocyanines (Pcs) are aromatic macrocycles with great potential in the clinical context, namely for use as photosensitizers (PSs) in the photodynamic therapy (PDT) of cancer.\(^1\,^2\) In this therapy the combination of visible light, molecular oxygen and a PS is able to generate reactive oxygen species (ROS) which can lead to cell damage and activation of signaling pathways involved in cell death, resulting in tumor tissues destruction.\(^2\) Phthalocyanines, besides their high efficiency in the generation of ROS, have the advantage to absorb light in the red and near-infrared regions (600-800 nm) of the electromagnetic spectrum, allowing the treatment of deep tumors. However, Pcs are hydrophobic and have a non-selective accumulation in the tumor due to their low specificity to the cancer tissues.

Concerning the limitations of Pcs to be applied in clinical cancer settings in the PDT of cancer, we have chemically modified the core of the Pcs by their conjugation with \(\alpha\), \(\beta\) and \(\gamma\) cyclodextrins (CDs).\(^3\) CDs are carbohydrate units with strong potential to act as PS-delivery systems,\(^4\) since they provide water solubility, being compatible molecules with a rapid cellular uptake and specific recognition by lectin proteins, which play a key role in cancer-related biochemical pathways. The photophysical and photochemical studies demonstrated that the conjugates Pc-\(\alpha\)-CD and Pc-\(\gamma\)-CD are water soluble, fluorescent, efficient generators of \(1\)\(O_2\), photostable and able to interact with human serum albumin. The promising photophysical data of the conjugates have prompted us to validate them as PSs against human bladder cancer cells (UM-UC-3). The photobiological studies demonstrated interesting differences between the photoactivity of Pc-CDs that differ only in the CD nature. The lower photodynamic activity of the Pc-\(\beta\)-CD when compared with that for Pc-\(\alpha\)-CD and Pc-\(\gamma\)-CD was attributed to its lower solubility in water, leading to a lower efficiency to generate ROS inside the cells. The promising photoactivity of Pc-\(\alpha\)-CD and Pc-\(\gamma\)-CD ensure their potential candidacy as PDT drugs.


Matrix Metalloproteinases as a therapeutic target in Hematological Neoplasias

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Keywords: Matrix metalloproteinases; Hematological neoplasias; Metalloproteinases inhibitor

The bone marrow microenvironment is the main support of normal hematopoiesis, although it is also important in processes like formation, maintenance and development of the neoplastic clones. The matrix metalloproteinases (MMPs) are an important player in bone marrow microenvironment, since they can degrade all the protein components of the extracellular matrix. On account of that, several studies show that MMPs are also involved in cancer development and progression. The majority of actual therapeutics for hematological neoplasias fails in some patients leading to relapses. Therefore the use of MMPs inhibitors may become a new therapeutic approach for these patients.

The aim of this study was to evaluate the therapeutic potential of Batimastat (BB-94), a matrix metalloproteinases inhibitor, in in vitro models of hematological neoplasias.

For this purpose, we used four hematological neoplasias cell lines, two Acute Promyelocytic Leukemia cell lines, the NB-4 and the HL-60 cells, the first one with the translocation t(15;17) and the second without this translocation, one Multiple Myeloma cell line, the H929 cells, and one Mielodysplastic Syndrome cell line, the F-36P cells. All cell lines were cultured in absence and presence of different concentrations of Batimastat (BB-94) ranged from 0,1 µM to 10 µM, in daily or single dose administration. To evaluate the effect of this inhibitor on cell viability and cell density we used the Trypan Blue Assay. Cell death was determined by optical microscopy (May-Grünwald Giemsa staining), and by flow cytometry (FC) using the Annexin V and Propidium Iodide double staining. It was also evaluated the activation of caspases using the Apostat probe.

Our results showed that BB-94 reduces cell viability and proliferation in a time, dose and cell line dependent manner. We found that the half maximal inhibitory concentration (IC_{50}) at 48 hours of exposure was, approximately, 2,5 µM for NB-4, 7,5 µM for HL-60, and 10 µM for H929 and F-36P. Besides that, the daily administration schedule seems to be more effective in the reduction of cell viability and proliferation when compared to the same doses in single administration. BB-94 induced cell death by apoptosis with activation of caspases, in a dose-dependent manner. The cytotoxic effect of BB-94 seems to be more active in the APL cell line with the chromosomal translocation.

In conclusion, our results suggest that BB-94 is a potential new targeted therapy in hematological neoplasias. However, therapeutic efficacy may depend on the cell type and genetic characteristics of the neoplasia, as well as the therapeutic schedule used.

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# P31

Is there a synergistic effect between ascorbic acid and conventional chemotherapy in the treatment of colon carcinoma?

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Keywords: Colon cancer; ascorbic acid; chemotherapy; synergistic effect

Background: Colorectal cancer (CRC) is a major health problem with more than one million new cases diagnosed worldwide every year. Ascorbic acid (AA), the reduced form of vitamin C works as a pro-oxidant at pharmacological concentrations, promoting the formation of reactive oxygen species which can induce cancer cell death. It has been proved that AA doesn’t protect cancer cells from chemotherapy but plays a protective role in normal cells. At the same time AA can enhance tumor growth inhibitory effect conferred by usual therapies regardless tumor type. Giving the positive feedback in turn of the use of AA with chemotherapy, the aim of this study is to evaluate the therapeutic potential of AA in combination with 5-Fluorouracil (5FU) in human CRC cell lines.

Methods: WiDr, C2BBe1 and LS1034 (multidrug resistant) cell lines were cultured in appropriate culture medium and incubated in absence and with different concentrations of AA and 5FU alone or in combination during different periods of time. The half maximal inhibitory concentration (IC50), as well as the interaction index were calculated after 48, 72 and 96 hours by sulphorhodamine B (SRB) assay. To evaluate cell survival clonogenic assays were done, the number of colonies was counted, being plate efficiency and survival factor determined. To evaluate cell viability and types of cell death, flow cytometry was performed, using annexin V and propidium iodide double staining.

Results: Results obtained with SRB showed an anti-proliferative effect induced by AA in all cell lines in a dose and cell line-dependent way ($r^2$>0.91). C2BBe1 cells revealed to be the most sensitive to AA (IC50=0.82mM) comparing to WiDr (IC50=5.9mM) and LS1034 cells (IC50=5.37mM). Clonogenic assays revealed that, as the concentration of AA increases, survival factor decreases. AA also induces a cytotoxic effect when present in higher concentrations. When in combination, we reached an additive effect at 72 hours of incubation for C2BBe1 and LS1034 and a synergistic effect at 96 hours in all cell lines, more evident in LS1034. Clonogenic assays corroborate these results.

Conclusions: Our study allowed us to verify the existence of synergism between AA and 5FU as the combination of these drugs induced an anti-proliferative effect and a decrease in cell survival more relevant than that obtained with compounds alone that probably affect somehow LS1034 resistance. The data obtained could contribute to the development of a promising therapy in CRC with reduced doses of conventional chemotherapeutic drugs and consequently, a decrease in secondary effects.
Genomic profile analysis in human lung cancer

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Keywords: Lung Adenocarcinoma; Squamous Cell Lung Cancer; MLPA.

Lung Cancer (LC) is the most common malignancy in the world and the leading cause of cancer death. Squamous cell carcinoma (SCLC) and adenocarcinoma (ADC) are the two major histologic types of non-small cell lung cancer (NSCLC). The detection of these tumors in early stages and the identification of genetic characteristics associated with a high risk of recurrence or with the prediction of clinical progression, remain challenging issues in clinical practice. Therefore, the characterization of the genomic profile of LC can contribute to the development of diagnostic solutions, targeted and personalized treatment, ultimately seeking to reduce the number of deaths from lung cancer and health costs associated with this disease. The main goal of the present study was to identify the genetic profile of LC by Multiplex Ligation-dependent Probe Amplification (MLPA). To achieve this purpose 44 lung biopsies: 22 non tumor and 22 tumor (45% classified as ADC and 55% as SCLC) were acquired. The MLPA assay is a simple, fast, reproducible and cost effective method for the analysis of multiple samples in parallel. The analysis using X050-A1 Lung Cancer probe panel (MCR-Holland, Amsterdam), allowed to genetically distinguish ADC from SCLC, being SCLC the one that showed a larger number of genetic imbalances. Alterations in gains and losses of genes PIK3CA and PTEN, respectively, seem to be characteristic of SCLC. Moreover, in SCLC we also detected frequently losses in PTEN and gains in PIK3CA and FGFR1 genes. In ADCs it were not detected statistically significant differences that allow the discrimination of each histological type. In addition, the FKBP8 gene presented the highest percentage of gain, revealing its potential role as a biomarker of LC. Moreover, the MLPA probe panel, specific for LC, seems to be efficient to identify changes in several genes associated with LC.
#P33

Oral tumors: from genomic imbalances to clinical personalized medicine - Are We Ready?

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Keywords: Oral tumors; Genomic imbalances; Clinical personalized medicine

Introduction: The overall incidence of oral cancer seems to be rising worldwide: for 2013, 36 000 new cases and 6 850 deaths are estimated, including lip, oral cavity, and pharynx cancer.1 Since the oral tumors display a great genetic heterogeneity with alterations in almost all chromosomes until now only few genes have been associated with oral carcinogenesis process. Thus, the identification of genetic markers associated to these tumors is essential in order to improve diagnosis, prognosis, early detection of tumors and relapses and ultimately to delineate individualized therapy. Methods: The genomic profile of 27 oral tumors was identified through the application of whole genome array-Comparative Genomic Hybridization (aCGH) technique. Additionally, Multiplex Ligation-dependent Probe Amplification (MLPA) technique was conducted in primary oral tumor samples with corresponding resection margins (macroscopically tumor-free tissue) allowing, in both types of tissue, the evaluation of a total of 133 genes previously associated to human malignancies.

Results: We verified that in tumor tissue the genes with the highest number of gains were mapped on chromosomal arms 3q, 6p, 8q, 11q, 16p, 16q, 17p, 17q and 19q, moreover, the genes with the highest number of losses were mapped on chromosomal arms 2q, 3p, 4q, 5q, 8p, 9p, 11q and 18q. Thus, several genes were highlighted as important for oral tumor progression. Additionally, the analysis between tumor and matched macroscopically tumor-free tissue allowed us to build a model of logistic regression in order to predict the kind of tissue. In this model, TUSC3 gene presented statistical significance, thus losses in this gene can be a good indicative of malignancy. Conclusion: Our results point towards the usefulness of several genes mapped in the aforementioned chromosomes as possible genetic markers in clinical practice.

# P34

**Genetic analysis of urine samples. A powerful and helpful tool in Bladder Cancer diagnosis and follow-up?**

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**Keywords:** Bladder cancer, genetic alteration, urine, MLPA

**Introduction:** Bladder cancer (BC) is one of the most common malignancies of the urinary tract being the fourth most common neoplasia in men and the fourteenth in woman, accounting for 5% of all malignancies in Europe. Bladder tumors have high recurrent rates (50-80%) that justifies a long and regular follow-up. However, noninvasive diagnostic strategies have low sensitivity and specificity or cystoscopy is very invasive and painful. This justifies the increasing use of urine to study BC cells since it is a non-invasive procedure. BC is characterized by a high chromosomal instability. The main goal of this study was to evaluate the concordance of the genetic profile of BC biopsies and the profile in urine, contributing to a noninvasive strategy in detection of BC.

**Methods:** In this study, 18 bladder biopsies (14 from patients with BC; 4 from patients without BC – control samples) were received from “Serviço de Urologia e Transplantação Renal do Centro Hospitalar e Universitário de Coimbra” and DNA extraction was performed. DNA extraction from 16 urine samples (the same patients) was also done. All samples were analysed by Multiplex Ligation-dependent Probe Amplification (MLPA) and the results were compared.

**Results and Discussion:** High DNA purity and integrity for urine sample were achieved by performing a pre-treatment, before the application of commercial extraction kit. The number of genetic alterations (gains and losses) was detected by MLPA in DNA extracted from BC biopsies was higher than in urine. Comparing genetic imbalances between BC biopsies and urine, there are some concordant alterations with statistical significance namely for ROBO1, ARL13B, NOS2 and CCND1 genes. The use of this biofluid in diagnosis and follow-up could be an important improvement in development of a noninvasive strategy.
#P35

**Targeted therapy in Bronchial-Pulmonary Adenocarcinomas: EGFR, KRAS Mutations, MET Amplification and ALK Rearrangement**

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**Keywords:** EGFR, MET, ALK, KRAS, Bronchial-Pulmonary Carcinoma

**Introduction**

ALK encodes a tyrosine kinase receptor and its rearrangement-ALK + was found in 2-7% of lung carcinomas, resulting a constitutively active and oncogenic protein reported as exclusive of EGFR and KRAS mutations and associated with resistance to EGFR inhibitors but highly sensitive to treatment with ALK-inhibitor crizotinib.

**Material and Methods**

Sections of 126 bronchial-pulmonary carcinomas surgical specimens of all histological types were screened for ALK positivity by FISH with break-apart dual color probe (Abbott) and immunohistochemistry (IHC) - monoclonal antibody clone 5A4 (Leica); EGFR and KRAS mutations were determined by DNA direct sequencing and EGFR and MET genes amplification, by FISH (Abbott).

**Results**

IHC was applied in FISH ALK+ cases and of the 126 screened tumors, 9 adenocarcinomas (7%) were FISH ALK +, 3+ and 2+ score in IHQ. Among these 9 FISH-ALK+ cases, 3 had EGFR mutations and 2 had both EGFR and MET gene amplifications in FISH; KRAS was wild type.

**Discussion**

IHQ correlates with FISH for ALK gene rearrangement, not excluding EGFR and MET alterations. ALK status has to be tested in advanced bronchial-pulmonary adenocarcinoma, as it could be responsible for TKI-resistance of EGFR mutated tumors that benefit from ALK-targeted agents. EGFR mutations search revealed again to be necessary in this context of targeted therapy.
Epigenetic modifications in Hepatocellular carcinoma - Can epigenetic modulating drugs play a role on HCC therapy?

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Keywords: Hepatocellular carcinoma; Epigenetics; DNA Methylation, Histone Code.

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer related deaths, with the heaviest burden on Southeast Asian and African countries, due to high rates of chronic Hepatitis B Virus (HBV) infection. The incidence of this tumor on Occidental countries is rising, essentially related to chronic liver diseases as alcoholic cirrhosis and chronic Hepatitis C Virus infection.

Epigenetics refers to heritable and reversible alterations on gene expression by regulatory mechanisms such as CpG island methylation, Histone Deacetylation and non-coding RNAs interference. Lately, epigenetic modifications have been pointed in HCC development through Tumor Suppressor Gene silencing, oncogene activation and chromosomal instability. Following this idea, it is thought that Epigenetic modulating drugs may constitute a therapeutic option for HCC.

By using a methylation-specific PCR protocol, we were able to find epigenetic alterations on some cell cycle regulator and apoptosis related genes (p16, DAPK and PTEN) on three different HCC cell lines. Additionally, we proved the therapeutic efficacy and synergism of Trichostatin (a histone deacetylase inhibitor drug) and Decitabine (a hypomethylating drug) on reducing cell viability on HCC cell lines evidenced by Alamar Blue reduction assay. We also observed the reversal of promoter gene methylation after drug treatment.

This study reinforces the theory that epigenetic modifications are involved in Hepatocarcinogenesis and shows that epigenetic modulating drugs may be useful on HCC treatment. This work is financed by Calouste Gulbenkian Foundation and Center of Investigation on Environment, Genetics and Oncobiology (CIMAGO), Faculty of Medicine.
Abstracts

Cell Biology


### #P37

**Cx43 deubiquitination regulates the degradation of gap junctions through macroautophagy**


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**Keywords:** Cx43, Ubiquitin, AMSH, UBPY, Macroautophagy

Gap junctions are specialized cell-cell contacts formed by connexins, which provide direct intercellular communication between eukaryotic cells. Although connexin channels may be regulated directly by the opening or closure of the channel pore, connexin internalization and degradation also plays a pivotal role in the regulation of gap junction intercellular communication. In the past few years, Cx43 ubiquitination has been implicated in the modulation of Cx43 internalization and its subsequent sorting through the endolysosomal pathway for degradation in the lysosome. More recently, it was demonstrated that Cx43 ubiquitination also targeted the protein for degradation by macroautophagy through a process mediated by the endocytic adaptor Eps15 and the macroautophagy adaptor p62. Moreover, we have also recently described that Cx43 ubiquitination can be reversed through the action of the deubiquitinating enzyme AMSH, which protects the protein from internalization and subsequent degradation. Here we investigate the role of Cx43 deubiquitination in the regulation of Cx43 degradation by macroautophagy through the use of dominative negative mutants and siRNA silencing of AMSH and the deubiquitinating enzyme UBPY. The results observed in this study suggest that Cx43 deubiquitination protects the protein from degradation by macroautophagy.
E3 ligase STUB1 and Lys63 ubiquitin chains in hypoxia: the degradation of HIF1A by Chaperone-Mediated Autophagy.

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Keywords: Hypoxia, Starvation, Autophagy, Ubiquitination

HIF1A (Hypoxia-Inducible Factor 1 alpha) is a transcription factor responsible for the expression of numerous hypoxia-responsive genes, critical in ensuring cell survival under low oxygen. Recently, we showed that HIF1A is a substrate for chaperone-mediated autophagy (CMA), especially under starvation (in vivo) or nutrient deprivation (in vitro) conditions. However, the molecular mechanism by which HIF1A is degraded through CMA is yet to be fully elucidated. We show, for the first time, that an E3 ligase is needed for CMA substrate degradation. Indeed, dominant-negative mutants of the chaperone interacting ubiquitin ligase STUB1 prevent degradation of HIF1A by CMA. Also, STUB1 depletion inhibits HIF1A ubiquitination and degradation by CMA. Moreover, using linkage specific antibodies, we show for the first time, that CMA activation induces a rearrangement in the ubiquitin chains attached to HIF1A, from Lys48 to Lys63. In fact, overexpression of ubiquitin mutants that abrogate formation of Lys63 chains inhibits HIF1A ubiquitination and degradation by CMA. Overall, data obtained thus far unveiled a new role for STUB1, as well as for Lys63 ubiquitination, in the context of CMA-mediated degradation. The switch, from Lys48 to Lys63 chains, suggests that STUB1 might be the molecular determinant of Lys63 ubiquitination that is ultimately responsible for HIF1A shuttling to CMA. This new molecular mechanism is essential for HIF1A degradation by CMA but might also apply to other substrates, due to the promiscuous nature of the E3 ligase STUB1.
Amyloid-β peptide-induced deregulation of protein, redox and Ca2+ homeostasis in microvascular brain endothelial cells

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Keywords: Alzheimer’s disease; vascular brain endothelial cells; proteostasis; Ca2+ homeostasis; reactive oxygen species

Abnormal accumulation of amyloid-β (Aβ) peptide in the brain is a pathological hallmark of Alzheimer’s disease (AD). In addition to neurotoxic effects, Aβ also damages brain endothelial cells (ECs) and can thus contribute to the degeneration of cerebral vasculature, which has been proposed as an early pathogenic event in the course of AD able to trigger and/or potentiate the neurodegenerative process and cognitive decline. However, the mechanisms underlying Aβ-induced endothelial dysfunction are not completely understood. We hypothesized that Aβ impairs protein quality control mechanisms both in the secretory pathway and in the cytosol and also compromises Ca2+ and redox homeostasis, leading to brain EC dysfunction and death by apoptosis. Using the rat RBE4 cell line as an in vitro model of brain ECs, it was shown that toxic Aβ1-40 concentrations induce endoplasmic reticulum (ER) stress increasing the levels of markers of the ER unfolded protein response (UPR) in a time-dependent manner. Furthermore, the ubiquitin-proteasome system (UPS) and the lysosome-dependent autophagic protein degradation pathway were found compromised in Aβ1-40-treated ECs. Under these conditions, impairment of Ca2+ homeostasis occurred through alterations of regulatory mechanisms at the plasma membrane, ER and mitochondria. In ECs treated with toxic Aβ1-40 a significant increase in levels of reactive oxygen species (ROS) and depletion of antioxidant defences were also observed. Finally, Aβ1-40 was shown to activate mitochondria- and ER stress-dependent apoptotic cell death pathways, which occurred through caspase-dependent and -independent mechanisms. In conclusion, Aβ impairs protein quality control mechanisms in brain ECs leading to ER stress and impairment of the autophagic and proteasomal degradation pathways, concomitantly with deregulation of mechanisms involved in Ca2+ and redox homeostasis. Consequently, these cells are not able to counteract the deleterious effects of Aβ and die by apoptosis.

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**Autophagy induction inhibits the release of pro-inflammatory IL-1b-containing exosomes after inflammasome activation.**

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**Keywords:** Exosomes, IL-1b, inflammasome, autophagy, inflammation.

A growing number of diseases have been established to be linked to prolonged or exacerbated inflammation, often associated with increased levels of extracellular IL-1b. Hence, reducing this pro-inflammatory pathway has been suggested as a major target for protective therapies in these pathologies. However, finding an effective mechanism for the inhibition of IL-1b secretion remains a challenge in the field. It has been extensively demonstrated that this cytokine is exported from cells, upon activation of the inflammasome, through several unconventional secretory pathways, including via exosomes. These vesicles, which represent a specific subclass of membrane vesicles originated from the fusion of multivesicular bodies (MVBs) with the plasma membrane, have been isolated from many body fluids and were described to accumulate in association with a variety of human diseases. Exosomes are important vehicles for intercellular communication, since they allow long-distance delivery of effector cargo. Therefore, in the context IL-1b driven inflammation, it is reasonable to speculate that those vesicles would be crucial for the stimulation of distant cells and the amplification of immune responses. Besides their fusion with the plasma membrane resulting exosome release, MVBs can also interact with autophagosomes generating a hybrid organelle termed amphisome, which may subsequently fuse with lysosomes (forming an autolysosome) to degrade the incorporated material. Therefore, it is conceivable to suggest that stimulating the fusion with lysosomes constitutes a strategy to prevent exosome secretion. One of the mechanisms that is known to promote such fusion is autophagy. Taken together, this information led us to explore the role of autophagy induction as a modulatory mechanism for the release of IL-1b-containing exosomes by the monocytic cell line THP-1, after inflammasome activation. We started by demonstrating that activation of the inflammasome, after incubation either with LPS or LPS and ATP, in THP-1 cells up-regulates the secretion of exosomes containing not only IL-1b cytokine but also its mRNA. Moreover, incubation of unstimulated cells with these IL-1b-containing vesicles led to the amplification of the pro-inflammatory response without any other immune challenge. Finally, we found that in THP-1 cells, previously activated with LPS and ATP, the stimulation of autophagic activity by rapamycin was able to inhibit both release of exosomes and total levels of IL-1b (protein and mRNA) produced and secreted. According to our knowledge, the results obtained in this study show, for the first time, that induction of autophagy may have an anti-inflammatory effect by inhibiting the release and further spreading of IL-1b-containing exosomes. Altogether, the results obtained in this study constitute an important contribution for the study of mechanisms involved in inflammatory responses.
**To beat or not to beat: Detrimental autophagy contributes to gap junctions degradation in ischemic heart**

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**Keywords:** Connexin 43, Ubiquitin, Ischemia, Autophagy

In the heart, gap junction intercellular communication (GJIC), through membrane channels formed by Connexins (Cx), ensures efficient electric activation and action potential propagation, resulting in coordinated contraction. Several pathological conditions characterized by conduction block and arrhythmogenesis have been associated with dysfunction of GJ regulation. We have recently shown that GJIC can be regulated through autophagy-mediated degradation of Cx43-containing GJ, however the physiological relevance of such mechanism and its implications in pathological conditions remain largely unknown. However, in the heart, autophagy plays a dual role, being either protective or detrimental, depending on the nature, extent and severity of the stimuli or insult. The main objective of this study was to evaluate the effect of autophagy mediated degradation of GJ in the ischemic heart. To address this question, we used a rat heart Langendorff retrograde perfusion system. Control hearts were subjected to perfusion with Krebs-Henseleit buffer, while ischemia was induced by 30 min of no-flow. A 15, 30 and 60 min of further reperfusion (I/R) period was performed at the same flow rate used before ischemia. To investigate the involvement of autophagy in degradation of Cx43 triggered by ischemia and I/R, hearts were perfused with KH solution containing pepstatin A and NH₄Cl. The effect of ischemia and I/R on the total levels of Cx43 was evaluated by Western Blot, while the subcellular distribution of Cx43 was determined by confocal microscopy. The involvement of autophagy in the degradation of GJ was determined by the interaction between Cx43 and autophagy markers, such as LC3, p62 and Beclin 1, by colocalization and immunoprecipitation assays. The results obtained showed that activation of autophagy occurs in ischemia, and is further increased during I/R. On the other hand, the levels of Cx43 do not significantly vary during ischemia, but gradually decrease during reperfusion, being partially reverted in the presence of pepstatin A and NH₄Cl. Microscopy data showed that ischemia and I/R results in a dramatic subcellular redistribution of Cx43, with a decrease of Cx43 at the intercalated discs and an accumulation at the lateral membrane. Moreover, co-localization and immunoprecipitation assays demonstrated an increased interaction between Cx43 and autophagy markers during ischemia and I/R, in particular in hearts perfused with pepstatin A and NH₄Cl. Altogether, this data demonstrates that degradation of GJ protein Cx43 in I/R results from an increased activation of harmful autophagy.


#P42

**Ubiquitin induces interference in communication: Ubiquitination of Cx43 leads to Gap Junction degradation in ischemic heart**

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**Keywords:** Connexin 43, Ubiquitin, Ischemia, Autophagy

Efficient electric activation and action potential propagation in the heart largely depends on gap junction (GJ) channels, formed by connexins (Cx), localized at the intercalated discs (ID). Therefore, fine-tuning and maintenance of GJs in cardiomyocytes is essential for normal heart function. Several mechanisms have been implicated in the regulation of the amount of Cx43 at the plasma membrane. Results from our lab demonstrated that Nedd4-mediated ubiquitination of Cx43 signals internalization and degradation of GJ, mainly through autophagy. However, the physiopathological relevance of this mechanism has never been addressed before. Given the importance of autophagy and GJIC to maintain heart function, we hypothesized that heart lesion induced by ischemia relies on the increased Ubiquitin-mediated autophagy degradation of GJ present in cardiomyocytes. To address this question we used a cardiac cell line (HL-1), primary cultures of cardiomyocytes and the heart Langendorff perfusion system, either subjected or not to ischemia insult. After, we determined the total amount of Cx43, and its ubiquitination levels, and interaction with Eps15 and Nedd4, by immunoprecipitation assays. By confocal microscopy we investigated the subcellular distribution of Cx43 induced by ischemia, as well as its colocalization with endocytic markers, such as Eps15. The involvement of autophagy was determined by the interaction of Cx43 with autophagy markers, such as p62 and LC3. The results obtained in this study demonstrate that incorporation of ubiquitinated Cx43 in HL-1 cells induced the internalization and degradation of GJ. Moreover, ubiquitination of Cx43 and interaction with Nedd4 and Eps15 increased in cardiomyocytes subjected to ischemia. Strikingly, we showed that heart ischemia led to lateralization and dephosphorylation of Cx43, that was accompanied by an increased interaction with Nedd4 and ubiquitination of Cx43 localized at the IDs, suggesting that ubiquitination is the signal that targets internalization of GJ under ischemia. These observations suggest that changes in the phosphorylation profile of Cx43, induced by ischemia, are likely to be the initial “signal” responsible for the recruitment of Nedd4 leading to ubiquitination of Cx43. Moreover we showed that during ischemia Cx43 interacts and co-localize with the autophagy machinery, p62 and LC3, suggesting that ischemia acts as a stimuli for ubiquitination, internalization and autophagy degradation of Cx43, affecting GJIC and cardiac function.
Abstracts

Clinical Research
Pharmacogenomics Applied to Personalized Isoniazid Treatment

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Keywords: isoniazide; NAT2; tuberculosis; hepatitis

Background: Isoniazid (INH) is the most effective and widely used drug for tuberculosis. Treatment may be complicated by acute toxic hepatitis, with risk of fatal liver failure and need of life-saving transplant, even in preventive therapy. The identification of risk genotypes offers the possibility to personalize INH therapy and reduce of the incidence of hepatitis. INH plasma concentrations are highly dependent on metabolism via acetylation by the polymorphic enzyme N-acetyltransferase 2, encoded by NAT2 gene. NAT2 genotyping, allows the classification of individuals as “fast acetylators” (FA), intermediate acetylators (IA) or “slow acetylators” (SA), the latter with higher risk for hepatotoxicity. Polymorphisms in genes encoding other enzymes, like CYP2E1, GSTM1 or GSTT1 are also involved in INH metabolism, and in the case of GSTs, a role in the hepatocyte response to chemical-induced stress cannot be ignored. Genetic variants decreasing proteins involved in bile salt transport may also be implicated, as is the case of V444A missense polymorphism in ABCB11 gene.

Purpose: The aim of this study was to evaluate the contribution of polymorphism of genes NAT2, CYP2E1, GSTM1, GSTT1 and ABCB11, to the susceptibility of INH-induced hepatitis.

Materials and methods: A total of 109 treated tuberculosis patients from CDP (Centre of Pulmonary Diagnosis) of Coimbra and Venda-Nova were genotyped. Eleven polymorphisms of NAT2 were genotyped by sequencing; polymorphisms in CYP2E1 (rs6413432 e rs2031920) and ABCB11 (rs2287622) were analyzed by PCR-RFLP assay and homozygous for GSTM1 and GSTT1 deletions (GSTM1*0/*0 and GSTT1*0*0) were identified using a PCR multiplex assay.

Results: Clinical variables such as age, alcoholic habits or previous hepatitis, were not associated with the occurrence of INH-induced hepatitis. Slow acetylators (52.3%) identified by NAT2 genotyping were significantly more prone to develop hepatotoxicity (p = 0.01; OR = 3; 95% CI = 1.23-7.35). Polymorphisms of CYP2E1, GSTM1 and GSTT were not associated with the phenotype. For ABCB11 polymorphism, homozygous for variant Ala had increased risk of developing hepatotoxicity (OR = 2.1; 95% CI = 0.9-5), though not reaching statistical significance. This effect was more evident for females than for males (OR = 2.19; CI = 0.45-10.58). Population was in Hardy-Weinberg equilibrium for all polymorphism.

Conclusions: INH-induced hepatitis is a complex phenotype dependent on genetic and non-genetic factors. NAT2 genotyping is useful to establish a personalized INH treatment, avoiding overdose, a recognized risk factor for INH-induced hepatitis. More than 50% of patients may benefit from genotyping. Polymorphism in ABCB11 gene deserves further investigation, especially among women.
Surgical Treatment of Spontaneously Ruptured Liver Tumours – Case Series

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Keywords: Liver neoplasms, Rupture, spontaneous, Surgery

Introduction: Spontaneous rupture is a rare but potentially deadly complication of several types of liver tumours. Hepatocellular Adenoma (HCA) and Hepatocellular Carcinoma (HCC) are the most frequently primary liver tumours associated with spontaneous rupture. Metastases, although the most frequent liver neoplasm, rarely suffer this complication. The choice of therapy depends on the hemodynamic condition of the patient, number, location and size of the nodules, as well as the functional liver reserve. In selected cases the first option is resection, with Transarterial Embolization (TAE) and perihepatic packing as valid alternatives.

Objectives: Evaluation of the department’s experience in the surgical management of ruptured liver tumours

Patients and Methods:
Retrospective study of the patients treated in our surgical department. From a prospectively maintained data base of all patients operated on for liver pathology in our surgical department in the period between January 1st 1988 and December 1st 2012, 9 patients underwent surgery for ruptured liver tumours. Six were male and three female. Median age was 54.2 (range 44-69). Most frequent clinical presentation was abdominal pain (eight cases), with hemorrhagic shock in four. The most frequent pathological finding was HCC (four cases), followed by HCA (three cases) and metastases (two cases). Mean dimension of the tumours was 8.2 cm (range 3.5 to 7 cm). Statistical analysis was performed with SPSS Statistical Package for the Social Sciences – IBM, version 21.0.

Results: Surgical resection was performed on all but one patient, who underwent perihepatic packing. Hepatectomy consisted of major liver resection one case and minor hepatectomy in seven cases. One patients was bridged to resection with TAE and one with packing and TAE. Perioperative mortality was null but one patient died on the 10th postoperative day from multiorgan failure (11.1%). Major morbidity (Dindo-Clavien classes III and IV) occurred in 3 cases (33.3% - bile leakage with biloma in two cases and evisceration in one case). Statistical analysis demonstrated that the single factor associated with morbidity was the need for transfusion (p < 0.05). Metastases were the pathological subtype with the worst prognosis, associated with high mortality in the first year (p < 0.05).

Conclusions: Our experience shows that, in spite of the advances in interventional radiology, surgical resection is still a valid option in the treatment of ruptured liver tumours and it can be performed with acceptable morbidity and mortality.
Gene expression as a tool for evaluating tissue reaction to biomaterials

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Keywords: Synthetic bone graft; Xenogenous bone graft; Physico-chemical properties; Inflammatory reaction; Gene expression

Inflammation is a physiological reaction to an infection or tissue injury, which also occurs when biomaterials are implanted in a living host. Its implantation triggers a series of biological events that include acute and chronic inflammations, whose effects are important to better understand their impact in the host, depending on the material’s origin.

The aim of this work was to study the inflammatory reaction triggered by intramuscular implantation of two bone graft materials: a xenograft of porcine origin (Osteobiol™ Gen-Os) and a hydroxyapatite based synthetic material (Bonelike™) commonly used in the daily clinic, by histological evaluation and gene expression analysis, based in the transcripts number of five distinct target genes, crucial to the inflammatory process (IL-1β, IL-6, TNF-α, CCL3 and CCL2).

The graft materials were then implanted in the lumbar muscles of Wistar rats and the inflammatory response was evaluated through histological analysis, after one week of implantation.

For systemic gene expression analysis, blood was collected from the rat’s tail vein at the beginning of the experiment and at the moment of sacrificing all rats, with the aim of quantifying the transcripts of some inflammatory mediators (five target genes mentioned above).

The results showed that both grafts have quite different characteristics in almost all the evaluated properties. The in vivo response evaluated from the inflammatory infiltrates revealed that although both implants did not cause severe inflammation, the synthetic granules elicit a consistently more intense inflammatory reaction than that triggered by the granules of the xenogenous material, particularly in terms of collagen production and formation of fibrous capsule.

The transcript levels of CCL2 and IL-1β were significantly elevated in blood of mice implanted with the synthetic material and the xenogenous one, respectively.

Our results suggest that bone graft materials trigger a systemic response from the host, fact that is proven by the increase of inflammatory mediators in the blood. The synthetic bone graft showed a Th2 polarization, triggering significantly higher levels of CCL2 expression, the chemokine that activates macrophages. The xenogenous bone graft triggered higher levels of IL-1β, the cytokine that is responsible for lymphocyte activation.

Taken together, the in vivo data demonstrate that the inflammatory response appears to be different for the two biomaterials evaluated, with each one triggering the activation of different cell types. The xenogenous material, for an implantation time of 8 days, seems to be in an earlier phase of the inflammatory reaction when compared to the synthetic material, during the same time period.

Despite some limitations, this study is innovative and represents a significant scientific contribution in dentistry. Studies like this can bring some light to the understanding of complex biological and clinical behavior of bone graft materials.
#P46

**Neuropsychological and Saccadic markers of Progression and Severity in Huntington’s Disease**

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**Keywords:** Oculomotor Function; Neuropsychology; Huntington’s Disease

Huntington’s disease (HD) is a genetic neurodegenerative disorder that primarily affects the basal ganglia and the fronto-striatal circuitry. Oculomotor abnormalities have been described as one of the earliest symptoms presented by HD patients and have also been reported in premanifest gene carriers. This longitudinal study aims at understanding the evolution of oculomotor performance with disease progression and severity.

A comprehensive battery of neuropsychological tests was used to assess the overall cognitive functioning of the participants: 13 early stage non-demented manifest HD patients, 11 premanifest HD participants, and 18 healthy controls. An experimental paradigm was designed to assess oculomotor function. Participants had to complete four different horizontal saccadic tasks with an increasing executive and/or memory load: prosaccade, antisaccade, 1-or-2 back memory prosaccade and 1-or-2 back memory antisaccade. Data was recorded using an iViewX high-speed eye tracker. Success rate (percentage of error free trials), latency, directional and timing errors were calculated for each task. Early manifest HD patients exhibited deficits in several of the neuropsychological tests applied, namely in visual and verbal memory, executive function, attention, visual perception and verbal and non-verbal IQ domains. No significant differences were found between premanifest HD and control subjects in any of these measures, suggesting a similar overt cognitive baseline. Comparison of saccadic parameters between groups unveiled statistically significant differences. Early stage HD patients showed a significant decrease in the success rate and a significant increase in the percentage of directional and timing errors. A statistically significant difference was found for latency between the two clinical groups and controls - manifest and premanifest participants exhibited a faster response time compared to controls, especially in the more demanding saccadic conditions. Our first-year results suggest that the performance of early manifest and premanifest HD participants deteriorates when an executive or/memory load is added to the task. Moreover, the clinical groups appear to have deficits in goal oriented oculomotor behavior – with a trend towards more automated responses at the cost of timely decision-making. These data suggest that temporal saccade properties under distinct working memory loads can potentially mark the presence of early neurodegeneration in HD and could serve as quantitative measures of disease progression and severity.
Choroidal thickness in diabetic retinopathy: the influence of antiangiogenic therapy

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Keywords: Antiangiogenic agents, Choroid, Diabetic retinopathy, Macular edema, Optical coherence tomography

Purpose: To analyze the effect of anti-vascular endothelial growth factor agents (anti-VEGF) in submacular choroidal thickness (CT) of diabetic retinopathy (DR) patients.

Methods: Cross-sectional study, which included twenty-five DR patients (50 eyes) divided in 2 groups, according to DR stage and previous treatments: non-proliferative DR and diffuse diabetic macular edema (DME) in both eyes (OU), submitted to macular laser OU and anti-VEGF injection only in one eye (NPDR+DME group, n=11); and proliferative DR OU, treated with panretinal photocoagulation (PRP) OU and anti-VEGF injection only in one eye (PDR group, n=14). In the study visit, all patients underwent optical coherence tomography with enhanced depth imaging protocol. Choroidal segmentation was performed manually. The medium CT in central macular area (CCT) and the CT in centrofoveal B-scan were obtained automatically.

Results: The 25 eyes treated with anti-VEGF showed a reduction on CCT (p=0.002) and subfoveal CT (p=0.004), compared with the fellow eyes treated with laser only. Independent evaluation of PDR group revealed similar results (CCT p=0.02; subfoveal CT p=0.03). In NPDR+DME group, CCT was also significantly thinner in anti-VEGF treated eyes (p=0.04). A correlation between the number of injections and a thinner CT was found in this group (p=0.03) and in the evaluation of all eyes together (p=0.03).

Conclusions: Diabetic eyes treated with anti-VEGF agents have reduced CT.
#P48

**Patterns of Progression in Diabetic Retinopathy. Correlation between phenotypes and genotypes**

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**Keywords:** Diabetic Retinopathy; Progression; CSME; Phenotypes; Genotypes

**Purpose:** To establish a correlation between phenotypes of non-proliferative diabetic retinopathy (NPDR) progression (phenotypes A, B and C) and different candidate genes in type 2 diabetic patients.

**Methods:** A population of 307 diabetic patients with NPDR, followed-up during a 2 years prospective study was classified in 3 different phenotypes (A, B and C) of DR progression based on non-invasive methods, Color Fundus Photography (CFP) to assess microaneurysm turnover (MAT) and Optical Coherence Tomography (OCT) to measure Retinal Thickness (RT). Eleven genes were selected from a list of candidate genes (ACE, AGER, AKR1B1, ICAM1, MTHFR, NOS-1, NOS-3, PPARGC1A, TGFB1, TNF-a, and VEGFA) and their distribution were analyzed for the 3 different phenotypes.

**Results:** The distribution for the 3 phenotypes was respectively 48.1%, 23.2% and 28.7%. Different SNPs of genes ACE, ICAM1, NOS1, PPARGC1A and VEGFA were found to be associated with different phenotypes. Three SNPs were specifically linked to CSME development: ICAM1 rs1801714 and rs5498, and NOS3 rs3918227.

**Conclusions:** This study identifies new polymorphisms within the genes ACE, AKR1B1, ICAM1, NOS1, PPARGC1A and VEGFA significantly associated with retinopathy progression. The identification of these phenotypes-genotypes correlations opens new perspectives for the management and the treatment of DR in type-2 diabetic patients.
Implication of low HDL-c levels in patients with average LDL-c levels – focus on oxidized LDL, large HDL subpopulation and adiponectin

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Keywords: Low-HDL-c levels; cardiovascular risk patients; oxidized LDL; HDL subpopulations; adiponectin.

Dyslipidemia is recognized as one of the major risk factor for the development of cardiovascular disease (CVD), which is a major clinical problem worldwide. Large prospective cohort studies has been recognizing the importance of reduce major risk factors, including cholesterol levels, in particular LDL-c, as a pivotal strategy to prevent the development/evolution of CVD and related events. However, it is now accepted that the current lipid-lowering therapies, in particular those directed to reduce LDL-c levels, such as statins, are insufficient to prevent part of the CV events; indeed, residual CV risk remains elevated even in clinical trials in which LDL-c levels have been aggressively reduced. It has been suggested that monitoring the type of HDL particles, rather than their total quantity, is a more reasonable way of determining the CV risk.

This study aimed to evaluate the impact of low levels of HDL-c in patients with LDL-c average levels, focusing on oxidative, lipidic and inflammatory profiles.

Patients with CV risk factors (n=169) and control subjects (n=73) were divided in 2 subgroups, one of normal HDL-c and the other of low-HDL-c levels. The following data was analyzed: BP, BMI, waist circumference and serum glucose Total-c, TGs, LDL-c, Oxidized-LDL, total HDL-c and subpopulations (small, intermediate and large), paraoxonase-1 (PON1) activity, hsCRP, uric acid, TNF-α, adiponectin, VEGF, and iCAM1.

In the control subgroup with low-HDL-c levels, significantly higher values of BP and TGs, and lower of PON1 activity and adiponectin, were found, vs control normal-HDL-c subgroup. However, differences in patient’s subgroups were clearly more pronounced. Indeed, low-HDL-c subgroup presented increased HbA1c, TGs, non-HDL-c, Ox-LDL, hsCRP, VEGF and small-HDL-c, and reduced adiponectin and large-HDL-c. In addition, Ox-LDL, large-HDL-c and adiponectin presented interesting correlations with classical and non-classical markers, mainly in the normal-HDL-c patient’s subgroup.

In a patient population with CV risk factors, low-HDL-c levels are associated with a poor cardiometabolic profile, despite the average levels of LDL-c. This condition is better viewed by non-traditional lipid markers, including HDL subpopulations (in particular large), Ox-LDL, as well as markers of inflammation and angiogenesis, such as hsCRP, adiponectin and VEGF. The existence of average HDL-c levels, with improvement of HDL quality-functionality, reduction of Ox-LDL and hsCRP and increment of adiponectin, might prevent the evolution of CVD in this type of individuals often identified as having “residual” CV risk. Proper pharmacological and non-pharmacological therapeutic interventions directed to raise HDL-c levels and functionality and to inhibit Ox-LDL levels are advisory preventive measures in this type of CV risk populations.

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#P50

**Comparison of visual function after bilateral implantation inferior sector-shaped near-addition and diffractive–refractive multifocal IOLs**

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**Keywords:** multifocal, cataract surgery, contrast sensitivity, mesopic, intraocular lenses (IOLs)

Manuscript accepted in August 21st 2013 for publication at the Journal of Cataract and Refractive Surgery.

**PURPOSE:** To compare visual function after bilateral implantation of multifocal Lentis Mplus LS-312 (Group A) or Acrysof Restor SN6AD1 (Group B) intraocular lenses (IOLs).

**SETTING:** Ophthalmology Unit, Centro Hospitalar e Universitário de Coimbra, and Visual Neuroscience Laboratory, IBILI, Faculty of Medicine, University of Coimbra, Portugal.

**DESIGN:** Comparative case series.

**METHODS:** Patients between 49 years and 76 years had bilateral cataract surgery with multifocal IOL implantation. Patients were evaluated preoperatively and 3 months postoperatively for distance, intermediate, and near visual acuities; static photopic and mesopic contrast sensitivity; and visual acuity under a glare source using the Metrovision contrast sensitivity platform. Color vision was evaluated with the Cambridge Colour Test.

**RESULTS:** Group A comprised 56 eyes and Group B, 44 eyes. Visual and refractive results were comparable between the 2 IOL groups. Photopic contrast sensitivity was significantly better in Group B at intermediate (2.2 cycles per degree [cpd] and 3.4 cpd) and high (7.1 cpd and 23.6 cpd) spatial frequencies. Under low mesopic conditions (0.08 candelas/m²), differences were significant at 1.1 cpd and 2.2 cpd spatial frequencies. There were no differences in visual acuity under a glare source or in color vision.

**CONCLUSIONS:** Both IOLs provided good distance, intermediate, and near visual acuities. Visual acuity under a glare source and color vision were similar in the 2 groups. However, photopic and low mesopic contrast sensitivities were better in Group B, particularly for intermediate spatial frequencies, which are important for night driving.
**HIV1 VPR POLYMORPHYSMS ASSOCIATED WITH AA 77: A VIRUS HOTSPOT?**

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**Keywords:** HIV1; Vpr; Mutation; Polymorphism

**Background and aims**

There are several HIV-1 Vpr polymorphisms that have been involved in biological functions of this viral protein that include replication and pathogenesis of the virus, with a direct influence in AIDS progression, namely in infants with perinatal acquired HIV-1 infection. The aim of this ongoing work was to study HIV1 Vpr polymorphisms at the position 77 in an HIV1-infected family, prompted by a clinical case of a perinatal-infected-5-year-old boy with repeated ear infections, but otherwise healthy.

**Methods**

Since July 2012, an HIV1-infected family (father, mother and son) has been studied in what regards HIV1vpr gene polymorphisms. The family members were clinically evaluated studying several clinical markers such as haematological parameters, viral load, development delay, opportunistic infections, and other pathophysiological conditions.

**Results**

The analysis of HIV1 vpr sequences revealed two different mutations at the sequence that codes for the amino acid position 77. Both parents were infected with a virus carrying a R77H mutation, while the child’s-virus had a R77Q variant. Both parents and child were considered asymptomatic, although the child had a very high viral load (1,073,899 RNA copies/ml). Following a therapeutic period to decrease the child’s viral load, the child continues with no clinical signs of disease and interrupts the therapy. The father developed minor symptoms, while the mother remains asymptomatic.

**Conclusions**

Our results show that different mutations associated with the aa 77 lead to non-progressive phenotypes. More, we identified a clinical case where the patient, a 5-year old child, remained with no visible signs of disease regardless of high viral load. With this study we aim to study Vpr not only as a bio-marker of disease progression, but also as an evolution flag of HIV1.
Abstracts

Genetics
CYP2D6 pharmacogenetics in the Portuguese Population – Clinical implications

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Keywords: CYP2D6, Metabolic profiles, pharmacogenetics

To conduct the medicine to a more personalized path it is necessary to combine Pharmacogenomic, Metabolomic and Proteomic analysis, which will provide the tools necessary to define a better treatment, with a pronounced clinical effect and minimization of adverse reactions. For that reason, drug metabolizing enzymes are deeply investigated, since they are very important in the detoxification process and xenobiotic metabolism, including drugs. CYP2D6 is an enzyme involved in the metabolism of various substances, such antihypertensives, opioids, antiarrhythmics, antidepressants and β-blockers. The coding gene is highly polymorphic, which influences enzymatic activity and originates a huge variability in the enzyme hydroxylation capacity. Presently, there are several haplotypes that define the enzyme activity as normal, null, decreased or ultrarapid.

With these haplotypes, it is possible to define four metabolic groups that corresponds to the enzyme in vivo activity: Poor Metabolizer (PM), IM (intermediate Metabolizer), EM (Extensive Metabolizer) and UM (Ultrarapid Metabolizer). Different metabolic profiles determine the processing of xenobiotics and endobiotics, thereby influencing disease risk, therapeutic efficacy and side effects, or toxicity to xenobiotics. Our work aimed to characterize the CYP2D6 pharmacogenetics and predicted metabolic profile in the Portuguese population.

The study comprised 300 Portuguese unrelated adult healthy volunteers. Genetic analysis included allelic discrimination and copy number determination with TaqMan® probes by real-time PCR. Allele duplications for CYP2D6*1, CYP2D6*2, CYP2D6*4 and CYP2D6*10 were further confirmed by PCR-RFLP.

Our results revealed 6.3% of individuals with PM metabolic profile and 4.7% with UM metabolic profile in the Portuguese population. It was also possible to verify that there is a European north-south gradient of metabolic profiles, probably caused by evolutionary pressure, including diet. These results are crucial to define a better clinical treatment and to avoid the adverse reactions. Future directions should include integration of basic and clinical aspects to define algorithms that could be applied in medical treatments.
Whole genome research by array-CGH– detection of copy number imbalances with clinical impact

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Keywords: array-CGH, copy number imbalances, genomics, clinical impact

Microarray-based comparative genomic hybridization (array-CGH), also called chromosomal microarray or molecular karyotyping, allows the possibility to screen the whole genome at once and with high resolution. It is now assumed that array-CGH should be the first genetic test offered to detect genomic imbalances in patients with intellectual disability (ID) with or without dysmorphisms, multiple congenital anomalies (MCA), learning difficulties and autism spectrum disorders (ASD). As array-CGH allows the detection of imbalances below the 5-10 Mb resolution level of conventional cytogenetics, the average diagnostic yield can be up to 10% higher. It also allows the detection of a large number of Copy Number Variants (CNVs) in these patients as well as in healthy individuals, which poses a great challenge in its interpretation. In addition to this increase in the detection of CNVs, the use of array-CGH in large cohorts of patients with ID, ASD and MCA has led to the identification of novel microdeletion and microduplication syndromes.

In our laboratory we have analysed nearly 1000 patients with ID, MCA and ASD by array-CGH. We have contributed to the identification of genomic imbalances in many of those patients that can justify their phenotype. Some imbalances are more frequent than others, and we have groups of patients that share the same imbalance, such as: 1q21.1q21.2, 16p11.2, 17p11.2 and 22q11.2 microdeletion/duplication syndromes, Xp22.31 duplication, ZNF41 gene deletion and IL1RAPL1 gene duplication. Opposite genomic imbalances are associated with mirror contrasting phenotypes such as macrocephaly/microcephaly, obesity/low body mass index. The impact on the phenotype of X-chromosome imbalances is quite challenging to interpret in females due to X-chromosome inactivation, as females with the same genomic imbalance can show different outcomes.

We have the knowledge and the expertise that allow us to study the human genome with high resolution, investigating the presence of CNVs, and offering the clinicians the possibility to correlate with the phenotype. In addition to array-CGH, we have technologies such as fluorescence in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) that allows the study of patients’ relatives. Like this, we can determine the origin of the alterations, the recurrence risk and offer prenatal diagnosis in future gestations.

Nowadays, the study of the human genome has a clear impact in the management of clinical genetics.
**#P54**

**Interference of host genetic profile in predicting response to chronic HCV infection**

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**INTRODUCTION:** In hepatitis C virus chronic infection, response to pegylated interferon α (PEG-INF) and ribavirin (RBV) combined therapy is successful in around 50-60% of patients. Recently, SNP rs12979860, located upstream of the IL28B gene, was identified as a genetic marker of response to PEG-IFN/RBV treatment. In ABCB11 gene, encoding the bile salt export pump protein (BSEP), the SNP rs2287622 (p.V444A, c.1331T>C) is now a well-established susceptibility factor for acquired cholestasis and recent evidence suggests that it also influences progression of viral hepatitis C. As new and promising, but highly expensive strategies, using HCV direct-targeting antiviral drugs are emerging, identifying predictive markers for individualized treatment is crucial. This work aimed to evaluate the correlation between these two polymorphisms and the response to INF-combined therapy.

**MATERIAL AND METHODS:** 117 patients with chronic HCV infection were included in this study. Peripheral blood was collected for both patient genomic DNA and viral RNA extraction. Genotyping of rs12979860 and V444A polymorphisms were determined by automated sequencing and RFLP assay, respectively. The viral load was assessed using the test COBAS® AmpliPrep/COBAS TaqMan® HCV® (Roche). The identification of hepatitis C virus genotype was performed with 2.0 VERSANT® HCV Genotype (LiPA) assay. Sustained virologic response (SVR), defined as undetectable levels of HCV RNA 24 weeks after completion of therapy, was considered the end point of treatment.

**RESULTS AND DISCUSSION:** For rs12979860, 12.8% of patients had the risk genotype, TT. Patients homozygous or heterozygous for the C allele had a significant increased probability of achieving SVR than patients homozygous for T allele (p < 0.01; OR = 6.1; IC95%: 1.88-19.79). As for V444A (T>C), almost 30% of patients were homozygous for C allele, associated with lower expression of BSEP, and only 15% were homozygous for T allele. No significant association was found with response to therapy, although patients with C allele achieved SVR less frequently than homozygous for T allele. Other markers of poor response were: HVC genotype 1 or 4 (p<0.003; OR-6.3; 95%CI-1.95 to 20.38) and absence of early viral response (EVR, reduction in serum HCV RNA levels by at least 2 log 10 IU / ml by week 12). Patients who did not obtain an EVR at 12 weeks of treatment, also did not obtain SVR. High risk genotypes 1 and 4 were identified in 67% of patients.

**CONCLUSION:** IL28B associated SNP, HCV genotype and EVR had a good correlation with the outcome of INF-combined therapy and may assist in the selection between PEG-INF-based or new targeted therapies. Larger samples are needed to better evaluate the role of ABCB11 V444A polymorphism. Prospective studies are the next step.
Mitochondrial genome analysis in frontotemporal lobar degeneration: tRNAs contribution

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Keywords: mitochondrial DNA; FTLD; mt-tRNA genes; sequence variations.

FTLD (frontotemporal lobar degeneration) the second most common early onset type of dementia. It is a heterogeneous neurodegenerative disease in many aspects, including clinical, neuropathological and genetic features, characterized by progressive changes in behaviour, executive dysfunction and/or language impairment with frontal and temporal lobar atrophy. Some patients present clinical and neuropathological overlap with Alzheimer’s disease (AD), suggesting similarities in pathophysiology, including mitochondrial DNA (mtDNA) involvement.

The aim of this study includes sequencing of 22 tRNAs mtDNA encoded in genes for identifying variations in FTLD patients, ascertaining their involvement in FTLD.

We investigated 70 patients, 39 females and 31 males, with probable diagnosis of FTLD (age range: 38-82 years, mean 63 ± 11) according to standard criteria recruited at Neurology Unit of the Centro Hospitalar e Universitário de Coimbra. Total DNA was extracted from peripheral blood and the analysis of the 22 tRNA mtDNA encoded genes sequences was performed by automated DNA Sanger sequencing and found variants were submitted to in silico analysis. A total of 28 different sequence variations were identified in 32 patients (46%). From these, 5 variations are probably pathogenic, according to the in silico analysis, all causing structure and binding minimum free energy changes: m.4435A>G is in a critical position and is totally conserved in all species studied; m.5772G>A is located in T-stem and leads to the disruption of Watson–Crick base pairing (C-G), being 100% conserved in all species; m.12166T>C alteration is in anticodon loop and has high percentage of conservation. The most frequent variation found is m.12308A>G, in the variable region of mt-tRNAleu2 conserved in all mammals tested; m.15946C>T variation has a high rate of conservation and it is located in the acceptor stem. Further investigation is needed to better understand the functional effect and alterations found and FTLD. However, this is an original study, being the first that investigate the sequence of the tRNA genes encoded by mtDNA in FTLD.
P2RX7, A new susceptible gene for Orthodontic-induced external apical root resorption

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Keywords: APICAL ROOT RESORPTION; ORTHODONTICS; P2RX7 GENE; POLYMORPHISM

INTRODUCTION: Orthodontic-induced external apical root resorption (EARR) is a complex phenotype, being determined by poorly defined mechanical and patient intrinsic factors. Although rarely serious, it results in permanent loss of the most apical dental structure and can affect the dentition longevity. Delineation of molecular profiles of EARR would unravel the mechanisms involved and facilitate prevention and more personalized orthodontic treatment planning. Polymorphisms of genes encoding proteins involved in alveolar and root remodeling have been implicated in EARR etiology. We proposed to study nine clinical and treatment related factors and polymorphisms of four candidate genes in order to construct a multifactorial integrative model to predict EARR.

MATERIALS AND METHODS: This retrospective study included 195 patients, 72 males and 123 females, with an average age of 17.24 years old (SD±6.8). Six teeth, the four maxillary incisors and the two maxillary canines, were evaluated. The average treatment time was of 36 months (± 10 months). EARR was evaluated by panoramic radiographs using a software prototype specifically developed for this work. Using a multiple linear regression model, where the dependent variable was the maximum % of root resorption for each patient, we evaluated the contribution of nine clinical variables and 4 polymorphisms of genes involved in bone and tooth root remodeling (rs1718119 from P2RX7, rs1143634 from IL-1B, rs3102735 from TNFRSF11B, encoding OPG, and rs1805034 from TNFRSF11A, encoding RANK). Genotyping was performed by RFLPs and real time TaqMan assays. To prevent interoperator error, all the measurements procedures were carried out by the same operator (S.A.P). The intraoperator error analysis was conducted by both the Student’s t-test for paired samples (for systematic error) and by the Dahlberg formula (for random error).

RESULTS: The most frequently affected teeth were maxillary incisors. EARRmax showed a mean value of 18% (±9.5%), a median of 17%, a minimum of 1.9% and a maximum of 49.7%. In this sample, clinical and genetic variables explained 30% of the maximum % of root resorption variability. The variables with more significant unique contribution to the model were: gender (P <0.05), treatment duration (P <0.001), premolar extractions (P <0.01), Hyrax appliance (P <0.001) and GG genotype of rs1718119 from P2RX7 gene (P <0.01). Age, overjet, tongue thrust, skeletal class II and the other polymorphisms had minor contributions.

CONCLUSION: This is the first study identifying P2RX7, a gene encoding a non-selective ion channel dependent on high levels of extracellular ATP with a pro-osteogenic effect, as a possible susceptibility factor to EARR. The five variables identified explained about 30% of phenotype variability, suggesting the existence of other etiologic factors.
CYP2D6 genetic variation and predicted metabolic profile in post-caesarean pain: pharmacogenetic interpretation

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Keywords: Caesarean, CYP2D6, Labor analgesia, Pain, Pharmacogenetics

The treatment of post-caesarean pain is essential for the early recovery and long-term post-partum. After caesarean, intravenous administration of morphine is a common procedure for pain relief. Pain is a complex process, in which the interaction of multiple genes and environmental factors influence the clinical efficacy of opioids. Morphines, the most widely used drug for the management of acute and chronic pain immediately following surgery. It activates μ receptor, leading to an upregulation of dopaminergic neurons, leading to an increase in dopamine release, deeply involved in pain control. CYP2D6 enzyme belongs to cytochrome P450 proteins family and is responsible for the oxidative metabolism of various drugs and endogenous substances, such as tyramine, a precursor of dopamine in the brain. CYP2D6 gene encodes this enzyme and is highly polymorphic, resulting in huge variability of metabolic activity, defining different metabolic profiles: poor (PM), intermediate (IM), extensive (EM) and ultra-rapid (UM) metabolizer.

The aim of this study is to perform the CYP2D6 pharmacogenetic characterization correlating with post-caesarean pain treated with morphine, in Portuguese Caucasian adult women. DNA was extracted from peripheral blood of 53 Portuguese Caucasian adult parturients in order to determine it and predict the metabolic profiles. Genetic analysis included allelic discrimination and copy number determination with TaqMan® probes by Real-Time PCR. Allele duplications were confirmed by long PCR and PCR-RFLP. The genotypes and metabolic profiles were investigated for association with pain score. The statistical analysis was performed by χ² test and the results are considered statistically significant if p<0.05.

A positive association was found between CYP2D6 reduced activity and pain. It can be hypothesized that if CYP2D6 activity is reduced, tyramine metabolism is decreased, resulting in reduced formation of endogenous dopamine. Consequently, the activation of signal transduction pathway that controls neuronal pain and analgesic effect may be reduced, leading to an increase of pain after caesarean.

The present work is a preliminary study and contributes to a better understanding of how the CYP2D6 allelic variants may affect pain sensation, after morphine treatment, with relevant impact in labor analgesia.
Personalized Periodontal Treatment – The role of Genetic test

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Keywords: Periodontitis, polymorphism, genetic test, personalized medicine

BACKGROUND Chronic periodontitis (CP) is an inflammatory disease of the supporting tissues of the teeth that represents the main cause of tooth loss in developed countries. Strong data support a role for genetic polymorphisms in the predisposition and progression of periodontal diseases, explaining the phenotypic variability observed amongst patients. Proinflammatory cytokines, interleukin-1α (IL-1A) and β (IL-1B), have been implicated in the immunopathology of chronic periodontitis (CP), being highly expressed in the crevicular fluid of patients. The presence of at least one allele 2 (corresponding to timine, T) in one of the two polymorphic loci, IL-1A -889 and IL-1B +3954, is considered to be a “risk genotype”, correlating with the levels of cytokines production and with the severity of CP. Null polymorphisms of genes encoding glutathione-S-transferase T1 (GSTT1) and glutathione-S-transferase M1 (GSTM1), two enzymes involved in detoxifying ROS and other cellular and xenobiotic metabolites, have also been associated with CP. Lack of functional activity of these enzymes may perpetuate the inflammatory process, leading to increased tissue damage. Genetic testing based on IL1A and B genotype has been increasingly used in clinical practice for personalized periodontal treatment planning, though no prospective studies have proved its value.

PURPOSE: Analyze the association of IL1A, IL1B, GSTT1 and GSTM1 genes polymorphisms with CP in a Portuguese Caucasian group and evaluate its utility as a genetic susceptibility test for CP.

METHODS: 32 patients with CP, identified according to established clinical criteria, and 81 controls were included in this study. DNA was isolated from saliva, using ORAgene™ kit (DNAgenotek). IL1A (rs1800587/-889) and IL1B (rs1143634/ +3954) polymorphisms were genotyped by RFLPs assay; GSTM1 and GSTT1 polymorphisms were studied by multiplex PCR.

RESULTS: For GSTT1, IL1A and IL1B variants, there were no statistically significant differences between patients and controls for any of the genotypes or allele frequencies analysed. Furthermore, expression of the “risk genotype” was also not associated with CP (p<0.05). A significant association was found between CP and GSTM1-null genotype (p=0.025; OR = 2.5, IC 95%: 1.1-5.9).

CONCLUSIONS: The lack of any association between the IL1 polymorphisms and CP, in the presented population, brings into doubt the usefulness of these candidate genes as markers of susceptibility to this form of periodontitis in the Portuguese population. Within the limits of this study, the GSTM1-null variant was statistically associated with CP, suggesting a role in the susceptibility to this disease. Larger population studies, and most of all, prospective studies, will be performed to evaluate the advantage of genetic testing in disease control.
# P59

**ASTHMA AND RHINOSINUSITIS: does genetic profile identifies who is getting which?**

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**Keywords:** Asthma, Rhinosinusitis, Genetic polymorphisms

**BACKGROUND:** Asthma and rhinosinusitis are airways inflammatory diseases, clinically heterogeneous and with a complex etiology, depending on multiple genetic and environmental risk factors. Rhinosinusitis is a common comorbidity in asthmatic patients and has been considered as a risk factor for its development and severity.

Genetic contribution for these phenotypes may account to 50-60%, so, genetic research can improve our understanding of their pathogenesis and allow identifying sub-phenotypes with different prognosis and response to therapy. Dissecting genetic susceptible profiles may also contribute to find new targeted therapies. An increasing number of susceptibility genes are currently being identified but the majority of reported associations have not been consistently replicated across populations of different genetic backgrounds. Genes encoding proteins involved in immunologic responses like IL4R, IL13 and IL17A, and proteins involved in the metabolism of ROS and environmental agents, like GSTP1, are natural candidate genes for these phenotypes.

**PURPOSE:** To evaluate whether rhinosinusitis and/or asthma in adults had common or different genetic profiles concerning polymorphisms of four genes: IL4R (rs1805015), IL13 (rs20541), IL17A (rs2275913) and GSTP1 (rs1695).

**METHODS:** 192 unrelated healthy individuals and 210 patients, 83 with rhinosinusitis and 149 with asthma (with or without associated rhinosinusitis), were studied. All polymorphisms were detected by real time polymerase chain reaction (PCR) using TaqMan assays.

**RESULTS:** Statistically significant association with asthma was observed for GSTP1 Ile/Ile genotype (Odds Ratio (OR) – 1.83 with 95% CI of 1.17 to 2.85; p = 0.008). The association sustains for allergic asthma (OR – 2.02 with 95% CI of 1.26 to 3.24; p = 0.004). The AA genotype of rs2275913 (IL17A) was associated with more than two-fold increase susceptibility to rhinosinusitis (OR – 2.31, 95% CI = 1.06 to 5.00, p = 0.024), and among patients, it also shows a stronger association with rhinosinusitis than with asthma (p = 0.006). There were no significant differences in the distribution of allelic and genotypic frequencies between patients and controls for the IL4R and IL13 polymorphisms analyzed. Hardy-Weimberg equilibrium was confirmed for all polymorphisms.

**CONCLUSION:** This study supports the existence of a significant association between GSTP1 Ile/Ile105Val polymorphism and susceptibility to asthma, but do not confirm the role of IL4R and IL13 polymorphisms. We describe, for the first time, an association between AA genotype of IL17 variant rs2275913 and the risk of developing rhinosinusitis. These results suggest that the genetic susceptibility profiles of asthma and isolated rhinosinusitis are not equivalent.
Abstracts

Neuroscience
Hemispheric asymmetries and snake threat detection in the human amygdala: an fMRI study

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Keywords: amygdala, snakes, hemispheric lateralization, threat detection, evolution

In humans, ecologically relevant stimuli (e.g. faces) are processed in central vision. Since central vision processes high-resolution details, it is better suited for identification processes that require accuracy. Peripheral vision is more likely to process objects that do only require coarse stimulus identification, such as animal shapes (e.g. snakes), due to its preference for low spatial frequencies and degraded colour information. A recent anthropological theory acknowledges differences between visual systems of primates who shared their environment with snakes (e.g. Old World monkeys) and those who have not (e.g. lemurs of Madagascar). This theory suggests that snakes acted as agents of evolutionary changes in the primate visual system, and might therefore have acquired a phylogenetic value related with the fear response. The amygdala is a core structure in the subcortical pathway for threat detection, and it seems to be preferentially entailed during automatic, preattentive detection of fear-relevant (e.g., snakes) as opposed to fear-irrelevant (e.g., rabbits) stimuli. This core structure in the learning of fear also shares many connections with the occipito-temporal visual pathway for object recognition, which is known to be biased to central vision.

Here, we used snake-related stimuli - snake faces, snake shapes, and control fake snakes - and manipulated both the spatial position and the allocation of attention to threat (implicit and explicit tasks). Twenty healthy participants have performed an event-related fMRI task. We hypothesized that snake faces should engage more the amygdala during central presentations, consistent with a central visual bias for face stimuli, but peripheral presentations of relevant shapes would dilute this central bias for detailed object processing.

First, we found larger amygdala responses to centrally presented snake stimuli (body, face or fake) compared to right peripheral presentations, independent of task and amygdala. Importantly, for peripheral presentations, a strong hemispheric lateralization was found, with stronger amygdala responses to real snake shapes (compared to fake shapes) when these stimuli were presented in the left hemifield (right hemisphere), but not for right hemifield presentations. This corroborates the hypothesis that real snakes have acquired a particular fear-relevant value in the evolutionary story of primates. This is also consistent with previous reports which show that attention to global cues and predator detection is right lateralized in vertebrates.

These results point to the role of central vision in primates, although not disputing the role of peripheral, less accurate, processing. In fact, left hemifield/right hemisphere (visual and emotional dominant) asymmetries found specifically for the (real) snake shapes in the amygdala suggest that these stimuli have phylogenetic value.
# P61

**Localization of secretases involved in the processing of β-amyloid precursor protein related to Alzheimer’s disease.**

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**Keywords:** Synapses; Alzheimer’s Disease; BACE1; ADAM10; Presenilin1

Although it is widely accepted that Alzheimer’s disease (AD) is a synaptic disorder and the amyloid-beta peptides (Aβ) formation is crucial for this pathology, the distribution of APP and of secretases (α- β- and γ) that mediated APP proteolytic processing in different nerve terminals remains to be clarified, nor is it known if their distribution changes in AD conditions.

This study aims to investigate the synaptic and subsynaptic (pre-, pos- and extra-synaptic zones) distribution of APP and secretases; ii) to define the presence of APP and secretases and their co-localization in different types of nerve terminals, mainly glutamatergic and GABAergic terminals; iii) to investigate whether AD condition affect the distribution of APP and secretases in nerve terminals. The synaptic distribution and density of APP, BACE1 (β-secretase), ADAM-10 (α-secretase) and presenilin -1 (PS1, a component of γ-secretase) were assessed through Western blot and immunocytochemical analyses in hippocampal synaptic and /or subsynaptic fractions obtained from adult male mice and also in a transgenic mice model of AD (3xTg-AD).

The data obtained showed that APP, α- and β-secretases were not enriched in nerve terminals (synaptosomes) as compared with the bulk of total membranes. It was also observed that APP was mainly located presynaptically, the α-secretase (ADAM10) was distributed pre- and extrasynaptically, whereas β-secretase (BACE 1) was preferentially located in the extrasynaptic fraction. APP and BACE 1 were partially co-localized by about 40% in hippocampal nerve terminals, and this secretase was present in higher levels in glutamatergic than in GABAergic terminals. In 3xTg-AD mice, we observed a clear decrease in ADAM 10 immunoreactivity, as well as an increase of PS1 in immunocytochemical analysis.

This study provides a first step to understand the particular susceptibility to dysfunction and degeneration of glutamatergic and GABAergic synapses in early AD, and might be useful to the development of novel therapeutic strategies.

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#P62

**Functional reorganization of the visual dorsal stream in Williams syndrome as probed by 3D visual coherence**

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**Keywords:** Dorsal visual stream, fMRI, Visual coherence, Williams syndrome

Object and depth perception from motion cues involve the visual dorsal stream which is known to be disrupted in Williams Syndrome (WS). This impairment has been reported in response to three-dimensional (3D) structure-from-motion (SFM) tasks and the electrophysiological neural substrates of such deficits were demonstrated to be distinctive in this clinical condition, providing evidence for functional reorganization. In SFM perception, motion and depth need to be first extracted in the dorsal stream to allow object categorization which is mediated by the ventral stream. Such interplay justifies the use of SFM paradigms to understand dorsal-ventral integration of visual information. WS represents a privileged model to investigate the nature of such processing because of its well known dissociation in dorsal (impaired) vs. ventral stream (relatively preserved) function.

In the current fMRI study, we assessed dorsal and ventral visual stream function in 7 WS (diagnosed through clinical and genetic examinations - FISH) patients (mean ± SE = 21.57 ± 3.01) and 9 control participants (mean ± SE = 21.22 ± 1.61). A performance matched 3D SFM visual integrative task in which motion cues drive 3D shape perception (faces v. chairs) was conducted. To better assess the nature and specificity of the neural substrates of 3D SFM perception in WS, high-level visual stimuli (static faces, places, objects and scrambled) and simple motion stimuli (2D coherent mobbing dots and static dots) were also presented to the participants.

We found evidence for substantial reorganization of the visual dorsal stream in WS with relatively spared ventral stream patterns, as assessed by whole brain ANOVA RFX (p<0.05, corrected for multiple comparisons). Individuals with WS recruited more medial regions (cuneus, precuneus and retrosplenial cortex) as compared to controls, who showed the expected dorsolateral pattern (caudal intraparietal sulcus and lateral occipital cortex/hMT+). Interestingly, this altered pattern of activation found in WS can already be identified in response to both low-level visual stimuli (static dots and 2D coherent moving dots) and static images of visual object categories (faces, places, objects and scrambled). Moreover, we observed that individuals with WS show activation of similar areas along the ventral stream as compared to controls, even though with a more widespread pattern of activation than the one found in the control group.

In sum, we found a substantial reorganization of dorsal stream regions in WS in response to 3D SFM, shape and motion perception, with a less affected ventral stream. Our results suggest the existence of a medial dorsal pathway allowing for information rerouting and reorganization in WS. This interpretation is consistent with recent findings suggesting the parallel flow of information in medial (in cuneus) and lateral parts (including hMT+) of the dorsal stream.
Caffeine modulates neuroinflammation and cell death in a model of retinal ischemia-reperfusion injury

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Keywords: Caffeine, adenosine A2A receptors, ischemia-reperfusion, retina, microglial cells

Caffeine is the most commonly used psychostimulant in the world and its effects are partly mediated by antagonizing adenosine receptors. Evidence suggests that caffeine attenuates inflammatory responses and affords protection upon CNS injury, by modulating adenosine A2A receptor (A2AR). Glaucoma is a neurodegenerative retinal disease and the second cause of blindness worldwide, characterized by optic nerve damage and retinal ganglion cell (RGC) death. It is known that microglia plays a key role in glaucoma and their overactivation leads to the production of proinflammatory mediators. Retinal ischemia-reperfusion injury (I-R) model mimics clinical situations such as acute glaucoma, and has been used to investigate retinal neuronal cell damage.

The aim of this work was to investigate whether caffeine intake prevents retinal neuroinflammation and cell death induced by ocular ischemia-reperfusion injury in rats.

Caffeine was administrated in the drinking water (1 g/l) two weeks before ischemia and until the end of the experiment. Retinal ischemia was induced in one eye by elevating the intraocular pressure for 60 min. Retinal reperfusion was reestablished, and the animals were euthanized 24 hours or 7 days after ischemia.

During 2 weeks of treatment caffeine intake was 125±7 mg/kg/day and serum caffeine concentration was 55 µM (2 weeks consumption) and 98 µM (3 weeks consumption). Moreover, intraocular pressure (IOP) can be since influenced by caffeine consumption, we measured IOP regularly. Caffeine did not affect IOP throughout the study.

At 24h post-ischemia, caffeine exacerbated microglia reactivity, without significant changes in the levels of TNF, IL-1β and IL-6. In addition, caffeine significantly increased the number of TUNEL-positive cells induced by I-R injury. However, at 7 days post-ischemia we found that caffeine decreased the effects of I-R on the number of active microglia, on the levels of TNF and IL-1β, and on the production of reactive oxygen species. Moreover, the number of TUNEL-positive cells in the retina was significantly decreased in caffeine-treated animals. Caffeine also prevented retinal thinning induced by I-R injury.

These results suggest that caffeine can modulate retinal neuroinflammation and it may afford neuroprotection to the retina against damage induced by I-R injury.

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Up-regulation of the density of adenosine A1 and A2A receptors in the prefrontal cortex from postmortem tissue of suicide completers

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Keywords: adenosine receptor, suicide, prefrontal cortex, postmortem

Chronic caffeine consumption attenuates the burden of repeated stress, the incidence of depression and suicide in humans and prevents phenotypic changes caused by chronic stress in rodents. Albeit the neurobiological basis of mood dysfunction is still ill defined, the involvement of pre-fronto-cortical circuits has been consistently reported, namely of Brodmann area 25 (BA25) in depression. The only known molecular target of caffeine in non-toxic doses is the antagonism of adenosine receptors and we have recently described an up-regulation of the adenosine neuromodulation system in the control of pre-frontal cortical function in animal models of attention deficit and hyperactivity disorders. Since 90% of suicide completers have clinical signs of depression, we now probed by Western blot analysis if there was also a modified density of adenosine A1 and A2A receptors (A1R, A2AR) in BA25 of suicide completers to justify targeting the adenosine neuromodulation system to manage mood dysfunction. We found an increase of the density of A1R (74.0±29.4%, n=6) and of A2AR (66.3±36.5%, n=6) in total extracts from BA25 of suicide completers compared to age-matched controls, using quality-validated (pH, RIN) postmortem fresh tissue samples. We next employed a subsynaptic fractionation, validated with adequate markers (syntaxin for presynaptic active zone; PSD-95 for postsynaptic density and synaptophysin for extra-synaptic zone), to determine if A2AR and A1R shared a common localization. We report that A2AR were predominantly located in nerve terminals outside of the active zone, although a minority was also located in the presynaptic active zone. In contrast, A1R were enriched in the active zone and in the postsynaptic density and in a lesser extent are located in the presynaptic active zone. These results show a different localization of adenosine receptors in the prefrontal cortex and confirm the up-regulation of adenosine receptors in the prefrontal cortex suicide completers, thus justifying the interest in targeting the adenosine neuromodulation system to manage mood disorders, namely depression.

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**Gender modulates type 2 diabetes progression and development of Alzheimer disease-like hallmarks in adult rat brains**

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**Keywords:** gender, brain, insulin, type 2 diabetes, Alzheimer disease

As gender differences become more unraveled, particularly at the molecular level, new insights are made in specific gender-dependent protocols for nutrition and also for therapeutics. Type 2 diabetes (T2D) is an epidemic that affects millions of people worldwide, being characterized by decreased insulin signaling and insulin resistance. Furthermore, this disease is also associated with aging and severe long-term complications such as encephalopathy, which in turn lead to cognitive deficits and increased risk of dementia (e.g. Alzheimer disease (AD)). Moreover, some reports showed that diabetes influences the levels of sex steroids in both plasma and central nervous system, although the mechanisms involved are not fully understood.

With this in mind, our hypothesis is that the complex interaction between normal brain aging and central insulin (INS)/insulin growth factor (IGF)-1-mediated signaling pathways dysfunction is differentially affected by gender (sex-specific hormonal pattern), altering the risk for the development of AD-like pathological hallmarks in adult T2D rat brains.

For this purpose, we analyzed (by using ELISA and immunoblotting methods) brain cortical homogenates from 8-months-old male and female Wistar and Goto-Kakizaki (GK) rats (a spontaneously, non-obese model of T2D). Both male and female GK rats showed higher blood glucose levels than age-matched Wistar cohorts. Regarding AD-like hallmarks, female brains presented significantly lower amyloid beta peptide ratio (Aβ1-42/Aβ1-40) as well as a decrease in BACE1 (Beta-secretase1) activity, whilst for the other AD hallmark, hyperphosphorylated Tau protein (P-Tau), an increase in P-Tau phosphorylated at Thr181 in female rat brains was observed. These results were accompanied by a relevant decrement of oxidative stress, in females compared to males, as seen by TBARS levels (lipid oxidation marker) and 8-hydroxydeoxyguanosine levels (DNA oxidation marker). In addition, we observed a general decrease in steroid hormone metabolism (from cholesterol to DHEA, testosterone and estrogen levels) in adult females, while progesterone levels showed no significant alterations in GK rat brains despite the slight increase in Wistar females compared to males. Interestingly, despite the decrement in brain estrogen, as well as in INS and IGF-1 levels in Wistar and GK female rats, an apparent compensatory mechanism was observed, given by the higher density of receptors (INS, IGF-1 and estrogen receptors) followed by the increase in phosphorylated Akt density.

In conclusion, despite the steroid hormone cascade imbalance observed in adult Wistar and GK female brains females seem to be more protected against the pathological changes associated with T2D and AD.

The dual facet of gamma oscillations

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Keywords: EEG Oscillations, Gamma-band Activity, Source localization, Simultaneous EEG/fMRI.

It remains an outstanding question whether gamma-band oscillations reflect unitary processes within the same task. EEG/MEG or localized cellular studies do lack the resolution or coverage to address the highly debated question whether single gamma processes are linked with many cognitive modules or alternatively with their own specific cognitive function, even in coherent perception tasks. One way to disentangle these issues would be to provide independent identification of their sources, using multimodal techniques. Here, we directly examined these questions by recording simultaneous EEG/fMRI from healthy volunteers performing an ambiguous perception paradigm requiring holistic integration. Data were acquired using a 64-channel MR compatible EEG system (NeuroScan, USA) in a Siemens Trio 3T MRI scanner and processed offline. MR related artefacts superimposed in EEG were attenuated by using template average subtraction and independent component analysis performed in Matlab®. BrainVoyager QX (Brain Innovation) was used for anatomical and functional data pre-processing and source estimation. EEG time-frequency features in the gamma frequency band were used as predictors for general linear model source localization. We found that distinct gamma frequency sub-bands reflect different neural substrates and cognitive mechanisms. Low gamma (<45 Hz) activity was tightly related to the decision making network, and in particular the anterior insula. High gamma (>55Hz) could be localized to early visual processing regions. The demonstration of a clear functional topography for distinct gamma sub-bands in the same task shows that independent gamma-band modulations underlie sensory processing and perceptual decision mechanisms.
Early changes in Ca2+-dependent CREB and ERK activity induced by oligomers of beta-amyloid 1-42 involve the activation of NMDA receptors

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Keywords: Abeta 1-42 oligomers; NMDA receptors; CREB; ERK; Alzheimer’s disease

Early cognitive deficits in Alzheimer’s disease (AD) are thought to be related to glutamate receptor dysregulation evoked by amyloid-beta peptide (Aβ). We previously showed that Aβ oligomers (AβO) 1-42 interfere with N-methyl-D-aspartate receptors (NMDARs) activation, leading to immediate intracellular Ca2+ (Ca2+ i) rise and a possible cascade of events at the synaptic level that may ultimately cause selective neuronal death. cAMP response element-binding protein (CREB), one of the main transcriptional factors involved in gene expression related to cell survival, memory formation and synaptic plasticity, has been shown to be altered in AD brain. Moreover, extracellular-signal-regulated kinase (ERK) is involved in a cascade of events that can also modulate the activity of CREB through phosphorylation of specific kinases. Importantly, CREB and ERK-associated signaling pathways are sensitive to Ca2+ i changes, and Ca2+ i dyshomeostasis has been largely described to occur in AD. Thus, in the present study we evaluated AβO1-42-induced initial changes in CREB and ERK activities and the role of NMDARs in mature cortical neurons (15 DIV). We show that AβO1-42 produce early Ca2+-dependent changes in phosphorylated CREB, reflecting CREB activity, along with a decrease in nuclear CREB levels upon prolonged Aβ exposure in cortical neurons, supporting a late decline in pro-survival functions of this transcription factor, and that these changes occur through the activation of NMDARs. A similar pattern of activation was observed for ERK, suggesting that both pathways can be connected in the response to AβO exposure. Interestingly, AβO1-42-evoked ERK and CREB activation were largely modulated by an antagonist of GluN2A-composed NMDARs, recognized to mostly present in synaptic sites. Overall, data support that early exposure to oligomeric Aβ1-42 exerts initial beneficial effects in mature cortical neurons through the activation of ERK-CREB signaling pathways, possibly linked to the activation of synaptic NMDARs.

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**The Result of Peripheral Retinal Degeneration on the Functional Organization of Visual Cortex**


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**Keywords:** Retinitis Pigmentosa, Retinal Degeneration, Visual Cortex, Functional Reorganization, fMRI

Retinitis Pigmentosa (RP) is an inherited retinal disease characterized by progressive degeneration of photoreceptors and consequent loss of peripheral vision and, in later stages, of central vision. The age of onset varies from infancy to adulthood, although the typical manifestations start at adolescence, making RP an appropriate way to study adult visual cortical plasticity.[1]-[4] This study aimed to determine the influence of rod-cone dystrophy on visual cortical function, by using functional magnetic resonance imaging (fMRI).

The brain images from four RP subjects (one male; mean age 49.50±12.23 years; mean disease duration 27.50±14.71 years) and seven age- and gender-matched healthy controls were acquired with a 3T magnetic resonance scanner and analyzed with BrainVoyager®. In the first part of stimulation, retinotopy was applied to delineate visual cortical areas. The second fMRI stimulus consisted in a random sequence of two checkerboard rings (covering central and para-central visual fields), during passive viewing and a one-back visual memory task. All participants were stimulated monocularly.

RP participants had statistically significant higher beta values than controls for visual area V3 dorsal (p=0.006) in the right hemisphere, and for visual area V1 ventral (p=0.042) in the left hemisphere.

This study demonstrated adult functional reorganization of visual processing in dorsal extrastriate and ventral striate cortex of RP patients, as suggested by previous studies[5]-[7]. Activation during task performance expanded into cortical areas representing the blind periphery in RP, even though the visual stimulation of central retina. We propose that attention might boost activity in peripheral representations under active task demands in RP, by feedback signals from higher order cortical areas into visual areas.

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Huntingtin regulates human neuronal stem cells division, a mechanism that is altered in Huntington’s disease

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Keywords: NSC, Huntington Disease, cell division

Huntington’s disease (HD) is a dominant neurodegenerative disorder caused by a CAG repeat on the coding region of HD gene. This gene encodes for a protein named huntingtin (Htt) that when mutated carries a polyglutamine (polyQ) expansion. Htt interacts with microtubules (MTs), directly with dynein and indirectly with dynactin through huntingtin-associated protein 1 (HAP-1) which binds to its p150Glued subunit to form the dynein/dynactin complex responsible for regulating microtubule-dependent transport of organelles in neurons. During cell division, Htt interacts with centrosomal region and MTs, ensuring a proper spindle orientation and regulating cell fate. Using human neural stem cells, in this study we showed that human Htt is essential to mitotic spindle orientation. Indeed, silencing Htt disrupted spindle orientation and mislocalized dynein, p150Glued and NuMA protein. We further addressed the effect of HD mutation on the role of Htt during spindle orientation in cells derived from HD patients. Mutant Htt with 50Q induced a dominant negative effect on spindle orientation. Although so far no therapeutic has been effective in HD, a promising approach involves interfering with the the levels of mutant Htt by recurring to posttranscriptional gene silencing mediated by small interfering RNAs (siRNAs). Resorting to this technique, allele-specific silencing of mutant Htt in HD cells was associated with the recovery in spindle orientation and with the localization of dynein, p150Glued and NuMA. Together, data suggest that wild-type and HD neural stem cells can be used as valid model systems to decipher molecular details regarding human Htt function and dysfunction in HD pathological situation.

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Investigation of plasma ATP levels in Frontotemporal Lobar Degeneration

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Keywords: Frontotemporal lobar degeneration, ATP, mitochondria, mitochondrial respiratory chain, energy impairment

In the last years, mitochondrial dysfunction and oxidative damage have been pointed as major contributors to neuronal loss in several neurodegenerative disorders, such as Alzheimer’s, Parkinson’s and Huntington’s diseases, Amyotrophic Lateral Sclerosis and, more recently, in Frontotemporal Lobar Degeneration (FTLD). The decreased activity of mitochondrial respiratory chain complexes will enhance ROS production and leading to a decline in ATP production, compromising key processes to cell maintenance and survival. Accordingly, we hypothesize that plasma ATP levels may be an indicator of the mitochondrial activity disturbance in tissues with high ATP demand, such as brain, possibly reflecting the energy impairment in the FTLD associated pathology.

The plasma ATP concentrations of 40 patients with probable diagnosis of FTLD followed in the Neurology Unit of the Centro Hospitalar e Universitário de Coimbra were determined using a bioluminescence technique and compared to an age-matched control group of 20 healthy subjects. Our results show that plasma ATP concentrations in FTLD patients are significantly decreased compared to controls, particularly in patients with cognitive impairment, according to MMSE scale evaluation. We have found a correlation between plasma ATP concentration and activity of complex I (positive) and ATP-synthase (negative), in lymphocytes of FTLD patients. These findings provide more evidence of low ATP production due to mitochondrial impaired activity in FTLD neurodegeneration. Additionally, since ATP may act as a signalling molecule, namely in glutamate release, it may play a role in neurochemical impairment occurring in neurodegeneration. These results are original and add a significant amount of data to the knowledge of mechanisms involved in FTLD pathogenesis.
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**NMDA receptor and Src related signaling in Alzheimer's disease**

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**Keywords:** Alzheimer’s disease, NMDA receptors, Src kinase, cortactin

Early cognitive deficits in Alzheimer’s disease (AD) are thought to be related to NMDA receptor (NMDAR) dysregulation and synaptic dysfunction in response to amyloid-beta peptide accumulation. In this study we analyzed age-and gender-dependent changes in NMDAR subunit protein levels and activation and Src related signaling in cortical and hippocampal homogenates from the 3xTg-AD versus age-matched WT mice. In the hippocampus, GluN2B Tyr1472 phosphorylation increased in 3xTg-AD females at 15 months of age, but decreased in 3xTg-AD males at 3 months of age; importantly, the latter was correlated with modified activity of Src Tyr kinase. Moreover, early decreased Src activation in the hippocampus and cortex of 3xTg-AD mice was associated with decreased Dab1 activation, a target of Src. Reelin protein levels were also diminished in the cortex of young 3xTg-AD mice. In addition, Dab1 and cortactin, two proteins linked to cytoskeleton stability, were significantly decreased in 3 month-old 3xTg-AD hippocampus and cortex. Our results evidence early reduced Src activity in the hippocampus, accompanied by decreased activation of Dab1 and GluN2B-composed NMDARs, favoring early NMDAR-related signaling modifications in AD.

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Adaptive Profile of Children with Autism spectrum disorder compared with non-autistic besides intellectual level

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Keywords: Autism spectrum disorder; Neurodevelopmental disorders; Adaptive behaviour; Functional profile; Vineland Adaptive Behaviour Scale.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that besides impairment in communication, social interaction and repetitive behaviour compromise adaptive functioning limiting social inclusion. The comorbidity with intellectual impairment is very common. However, the relevance of Intelligence Quotient (IQ) versus ASD specific deficits in acquisition of personal and social daily skills remains unclear.

The main goal of this study is to understand besides a well-known IQ relevance on adaptive behaviour acquisition the influence of specific ASD diagnosis on it. For that we have compared the adaptive behaviour between two population with neurodevelopmental disorders where the principal diagnosis were ASD versus other neurodevelopmental disorders (OND), matched for IQ and chronological age.

The sample consisted of 217 school-aged children (mean ± SD=147±36 months; 175M/42F): ASD (ADI-R/ADOS positive; N=115), OND (no clinical ASD criteria; N=102). These two groups were subdivided in four, taking into account the classification of intellectual disability (ID) of the CID-10 (ID=IQ<70), and matched by FSIQ: ASD with no ID [N=72]; ASD with ID [N=43]; OND with no ID [N=54]; OND with ID [N=48]. The functional profile by ID in communication, daily living skills, socialization and adaptive behaviour composite was compared. Statistical analysis was performed comparing the standard scores (SS) of Vineland Adaptive Behaviour Scale (VABS) within the two clinical groups and between the four subgroups. Significance level (α) = 0.05.

T-test showed significant differences between principal diagnosis (ASD versus OND) for daily living skills (DLS) (p = 0.021) and socialization (p = 0.036).

The comparisons in the four subgroups based on principal diagnosis and ID classification showed a differential profile on VABS. The ID subgroups differ significantly in DLS (p = 0.011), socialization (p = 0.040) and adaptive behaviour composite (p = 0.047); ASD group demonstrated lower SS as expected. In the subgroups without ID the same pattern of profile was observed, however difference only was significant in socialization (p = 0.026).

We can conclude that with VABS evaluation the impairment in adaptive behaviour within the domain of socialization skills remains a distinctive factor of ASD versus OND, independently of ID. However, co-occurring ID conditions result in further debilitating effects on overall functioning and adjustment, especially in ASD. These results have significant clinical and educational implications, enhancing the relevance to focus the intervention on teaching the daily live activities as early and intensively as possible to the ASD population.
Investigating the excitation/inhibition ratio in the pre-frontal cortex of subjects diagnosed with Autism Spectrum Disorder

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Key words: Autism Spectrum Disorder; GABA; Glutamate, Pre-frontal cortex

Individuals diagnosed with Autism Spectrum Disorder (ASD) show deficits in social interaction, verbal and non-verbal communication, and stereotyped behaviors. An imbalance in the brain excitation/inhibition regulatory dynamics has been proposed as a possible explanation for an abnormal neurodevelopment. The pre-frontal cortex is associated with executive functions, social cognition as well as inferring intention from others’ actions, which are known to be impaired in ASD. We used MEGA-PRESS to quantify GABA/tCr in the pre-frontal cortex of children, adolescents and adults with ASD (n=7; age range: 11 - 17; males), and controls (n=9; age range: 10 - 24; males). Additionally, PRESS (TR=2s, TE=30ms) was used to quantify Glutamate+Glutamine (Glx)/tCr levels in the same volume as MEGA-PRESS. With this approach we aimed at investigating the direction of an excitation/inhibition imbalance in this region in ASD. Glx/tCr was increased in the ASD group (p=0.008), while no differences were found for GABA/tCr (p=0.21).

These results show an increase in the excitation/inhibition ratio in the ASD group towards great excitation and/or deregulated glutamatergic metabolism. Combining the conventional PRESS sequence with the J-difference editing MEGA-PRESS has proven to be useful in clarifying the pathophysiology of ASD, showing that there is indeed an imbalance in a critical equilibrium. Moreover, knowing the direction of this imbalance may allow the development of further effective therapies and follow their effects on patients.
Antidepressant effect of treadmill exercise in mice submitted to a single neurotoxic dose of methamphetamine

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Keywords: methamphetamine, depression, anxiety, physical exercise, antidepressant.

Background: Recently we demonstrated that a single high dose of methamphetamine (METH) caused a long-lasting depressive phenotype and persistent frontal cortical dopaminergic deficits. Notably there is evidence to support the hypothesis that physical exercise alleviates depressive and anxiety symptoms. Thus we assessed the impact of treadmill exercise on long-term depressive- and anxiety-like behaviour in METH-exposed mice.

Methods: Herein, C57BL/6 mice were submitted to a treadmill exercise program (five days a week for seven weeks) starting 24 h post-single high dose of METH (30 mg/kg, i.p.). The depressive- and anxiety-like behaviour were assessed 7 weeks following METH injection by tail suspension and elevated plus maze tests, respectively. Frontal cortical dopamine (DA), tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP) and glial cell line-derived neurotrophic factor (proGDNF) levels were also assessed.

Results: METH triggered a depressive-like behaviour in the tail suspension test 7 weeks post-injection. Remarkably, this behavioural phenotype was reversed by treadmill exercise. However, METH did not induce either an anxiety-like behaviour or locomotor impairment in the elevated plus maze test. On the other hand METH triggered long-term frontal cortical DA/TH depletion which was not recovered by exercise. GFAP and proGDNF levels were not changed by both METH and exercise.

Conclusion: We present new evidence that a 7 weeks treadmill program provided an antidepressant effect to METH-intoxicated mice. This beneficial effect offered by the exercise did not associate with dopaminergic recovery. The literature proposes other mechanistic candidates, such as increased serotonergic transmission.

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Endoplasmic reticulum stress and tau protein hyperphosphorylation are related events during brain endothelial cell dysfunction

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Keyword: Endoplasmic reticulum stress; tau protein hyperphosphorylation; endothelial cells

The endoplasmic reticulum (ER) is the principal organelle responsible for the proper folding/processing of nascent proteins and perturbed ER function leads to a state known as ER stress, that mammalian cells try to overcome through a homeostatic set of protein signalling pathways and transcription factors termed the unfolded protein response (UPR). However, persistent and severe ER stress triggers an apoptotic cascade resulting in cell death. Recent studies have shown that UPR activation occurs in neurons with diffuse tau protein phosphorylation, suggesting a linkage between tau pathology and ER stress in Alzheimer’s disease (AD) and other tauopathies. Moreover, the Aβ peptide, which accumulates in brain parenchyma and in the walls of cerebral vessels of AD patients, induces UPR activation and triggers tau protein hyperphosphorylation, suggesting a link between ER stress, tau protein phosphorylation and Aβ deposition in this neurodegenerative disorder.

We hypothesized that ER stress and tau protein hyperphosphorylation are related events in AD leading to brain endothelial cells injury, which occurs in early stages of the disease and potentiates neuronal injury and cognitive deficits. To test our hypothesis, rat brain endothelial cells (RBE4) were treated with thapsigargin (TP) or brefeldin A (BA), two well-known ER stressors, or with okadaic acid (OA), a protein phosphatase 2A inhibitor. In TP-, BA- or OA-treated RBE4 cells a significant increase in the levels of ER stress markers and phosphorylated tau protein was found. Concomitantly, RBE4 cells survival was compromised and the apoptosis-related caspases -9 and -3 were activated. Interestingly, we observed that levels of the amyloid precursor protein (APP) and β-secretase (BACE) levels/activity were affected under ER stress and tau protein hyperphosphorylation conditions.

Results suggest that tau protein hyperphosphorylation can induce ER stress that in turn promotes the phosphorylation of tau protein and that this vicious cycle can contribute to the AD-associated cerebral amyloid angiopathy (CAA) and endothelial dysfunction.

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The electrophysiological signature of response caution

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Keywords: EEG/ERP, inhibitory control, reaction time, go/no-go task, impulsivity

In go/no-go tasks, participants are requested to respond to frequent go stimuli, while withholding the response to infrequent no-go stimuli. Different participants choose different response strategies. More cautious subjects respond slower in go trials thereby increasing the chances of successful response inhibition in no-go trials. Less cautious, more risk-taking subjects trade accuracy for speed: respond faster in go trials and, consequently, present a higher number of errors of commission (failures to inhibit the response in no-go trials).

Our aim was to characterize the neural correlates of the different response strategies associated with fast or slow responses in go/no-go tasks. For this, we recorded the electroencephalogram (EEG) of healthy participants and individuals with a neurodevelopmental disorder associated with impaired impulse control, Neurofibromatosis type 1 (NF1), during performance of a go/no-go task with constant inter-stimulus intervals and high go probability (89%).

In both groups, faster participants failed more often to inhibit the response in no-go trials. In addition, individuals with NF1 responded faster to go stimuli and committed a significantly higher number of errors of commission than control participants.

Analysis of pre-stimulus slow preparatory potentials revealed two regions that showed significant correlations with reaction time. Frontal scalp sites showed a positive correlation with reaction time, while lateral central sites, contralateral to the response hand, showed negative correlations. The lateral central activation resembles the pre-motor activation observed in cued paradigms. In line with their response style (faster, less accurate responses), individuals with NF1 presented reduced pre-stimulus frontal activation and increased pre-motor activation in comparison with control values.

Next, we investigated if these responses related not only with reaction time but also with the success of response inhibition in no-go trials. Comparison of correct withholds with commission errors revealed that reduced frontal pre-activation was associated with the error trials. No effect was found regarding motor pre-activation.

In conclusion, our data suggests that response caution evident in slow and accurate task performance is associated with the level of engagement of the frontal cortex during the preparatory pre-stimulus period.

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Resveratrol ameliorates motor coordination and mitochondrial function in Huntington’s disease models

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Keywords: Huntington’s disease, de(acetylation), mitochondria, resveratrol, nicotinamide.

Mammalian sirtuins are a conserved family of class III NAD+-dependent deacetylases, which have been postulated to be beneficial for targeting mitochondrial abnormalities. Being a neurodegenerative disease that selectively affects the striatum and the cortex, Huntington’s disease (HD) has been commonly linked to mitochondrial dysfunction as one of the main pathological mechanisms. Therefore, we hypothesized that modulation of sirtuins might be beneficial in HD.

In this study we tested the influence of resveratrol (RESV) versus nicotinamide (NAM, a Sirt inhibitor) in counteracting mitochondrial dysfunction in HD cell models expressing full-length human mutant huntingtin. Moreover, we analyzed the effect of RESV and NAM in in vivo HD models. Using striatal and cortical neurons isolated from YAC128 transgenic mice embryos and HD human lymphoblast cell lines, we observed a slight decrease in histone acetylation with RESV and increased histone acetylation with NAM. Moreover HD lymphoblasts exhibited a decrease in PGC-1alpha and TFAM protein levels, leading to a small reduction in the number of mitochondrial DNA copies. Functionally, both HD models displayed a deregulation in mitochondrial parameters, namely mitochondrial respiration and membrane potential, implicating a decline in mitochondrial function. Interestingly, both RESV and NAM were able to completely restore most of the evaluated parameters in vitro, providing a positive add on mitochondrial function in HD. In in vivo study 1 mg/kg/day RESV and 250 mg/kg/day NAM were administered to 9 month-old YAC128 versus wild-type (WT) mice during 28 days. We found that RESV increased the latency to fall off in rotarod test; moreover, RESV and NAM greatly increased histone acetylation in both YAC128 and WT striatal and cortical samples. Additionally, RESV increased the expression of mitochondrial-encoded genes in YAC128 cortical samples. These data suggest that RESV and NAM modulate protein acetylation and ameliorate mitochondrial function in HD, and that RESV partially controls HD-related motor disturbances.

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Activation of adenosine A3 receptor protects retinal neural cells from excitotoxicity-induced cell death

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Keywords: Adenosine; Neuroprotection; Retina; Glaucoma

Glaucoma is a degenerative optic neuropathy, characterized by retinal ganglion cell (RGC) death. Current treatments are limited to lowering intraocular pressure (IOP), but a significant number of patients continue to lose vision despite successful IOP control. Therefore, novel strategies to save RGCs and prevent vision loss are valuable.

Adenosine is a neuromodulator in CNS. The modulation of adenosine receptors (AR) activity has been shown to protect against a broad spectrum of brain insults. In this work, we investigated whether A3R activation prevents RGC death induced by excitotoxicity.

In mixed retinal cultures, exposure to kainate plus cyclothiazide (KA+CTZ) for 24h significantly increased TUNEL-positive cells as compared to controls. When the cells were pre-incubated with Cl-IB-MECA (1 µM), a selective A3R agonist, and then exposed to KA+CTZ, the number of TUNEL-positive cells was similar to control. When the cells were pre-incubated with Cl-IB-MECA and MRS1191 (1 µM), a selective A3R antagonist, or with MRS1191 alone, and exposed to KA+CTZ, the number of TUNEL-positive cells were not significantly different from KA+CTZ condition. By immunohistochemistry, we observed that RGCs express A3R. The potential neuroprotective effect of A3R activation was also evaluated in cultured retinal explants using propidium iodide (PI) assay. In control cultures, the ratio of PI-positive cells (DIV4/DIV2) was 1.31±0.10. Exposure to NMDA (300 µM) for 48h, significantly increased PI-positive cells ratio to 3.50±0.23. Pre-treatment with Cl-IB-MECA (1 µM) prevented the increase in PI-positive cells ratio induced by NMDA (1.17±0.06). In cultures treated with A3R agonist and antagonist and exposed to NMDA, the PI-positive cells ratio was not significantly different from NMDA condition.

Our results demonstrate that A3R activation is neuroprotective against retinal cell death. These results suggest that the targeting of A3R in RGCs may have great potential against RGC death, in diseases like glaucoma.

Autophagic-lysosomal pathway impairment in MCI and AD cybrids is driven by mitochondrial dysfunction-dependent microtubule network disruption.

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Mitochondrial abnormalities have been widely described in Alzheimer’s disease (AD) and mild cognitive impairment (MCI), an intermediate state before declared dementia. Moreover, the accumulation of autophagosomes (AVs) in AD brains suggests that macroautophagy is induced in AD brains but AVs transport and maturation is likely to be impaired. Clearance of AVs requires intact microtubules, so that these structures can go towards the cell body where the lysosomes are located.

Our goal was to demonstrate that mitochondrial metabolism changes observed in retinoic acid (RA) differentiated MCI and AD cybrids, alter microtubule dynamics, which impairs the removal of engulfed materials within AVs by the autophagic-lysosomal pathway (ALP). We observed that mitochondrial membrane potential and dynamics is impaired in both cellular models. Further we found a disrupted microtubule network that impairs ALP efficiency, namely by decreasing autophagic flux in the AD group. Interestingly, MCI cybrids have an increased autophagic flux, likely a compensatory response attempting to degrade materials that are accumulating within the cell.

Overall, we demonstrate that mitochondria dysfunction, potentiates a disruption of intracellular trafficking leading to cellular demise.
Physiological interplay between ecto-5’-nucleotidase and adenosine A2A receptors in nerve terminals of mice prefrontal cortex

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Keywords: Ecto-5’-nucleotidase; prefrontal cortex; synaptic transmission; adenosine receptors; nerve terminals.

The activation of adenosine A²A receptors (A²A R) is mediated by adenosine selectively originated from the extracellular catabolism of released ATP. Ecto-5’-nucleotidase (e-5’N) plays a key role in the formation of ATP-derived adenosine and in the subsequent activation of A²A R to control synaptic plasticity. Upon brain injury, ATP is released as a stress signal and both e-5’N and A²A R are up-regulated in parallel. This prompts the hypothesis that e-5’N and A²A R could be co-localized and co-regulated.

The present study aims to define: i) the synaptic and sub-synaptic localization of e-5’N in cortical regions of adult C57/Bl6 mice, ii) the co-localization of e-5’N with A²A R in slices from the prefrontal cortex (PFC) and in cortical nerve terminals, iii) if the genetic deletion of A²A R affects the density of synaptic e-5’N in cortical regions, and finally iv) the role of e-5’N in synaptic plasticity in the PFC. The comparison by Western blot analysis of the density of e-5’N in PFC total membranes and synaptosomes revealed that e-5’N, albeit present in significant amount in nerve terminals (52.1 ± 2.3%, n=4), was not enriched in PFC synapses. The fractionation of PFC synaptosomes unveiled the presence of two different isoforms of e-5’N, one being more present at the presynaptic and extra-synaptic fractions (~50 kDa) and the other (~70 kDa) at the post-synaptic fraction. The pull-down of A²A R revealed the co-immunoprecipitation of A²A R with e-5’N in the prefrontal cortex. However, the synaptic density of e-5’N was not affected in the PFC of global A²A R knockout mice. Furthermore, electrophysiological studies in PFC slices incubated with a selective antagonist of A²A R (SCH 58261) demonstrated that the activation of A²A R was necessary to obtain long term potentiation (LTP); however when e-5’N was blocked (with AOPCP), and consequently the adenosine formation was prevented, the basal synaptic transmission suffered an inhibitory effect but there were no significant changes in the LTP phenomenon. The results show that e-5’N is co-localized with A²A R in nerve terminals, thus supporting their functional interplay, but also indicate that e-5’N and A²A R are independently regulated, paving the way to consider e-5’N an independent target to manage neurodegeneration.

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**Specific EEG/ERP responses to dynamic facial expressions in virtual reality environments**

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**Keywords:** Dynamic Facial Expressions; EEG; ERP; Right Hemispheric Lateralization; Virtual Reality

Visual event-related potentials of facial expressions (FEs) have been studied using usually static stimuli after a nonspecific black screen as a baseline. However, when studying social events, the ecology of the environment and stimuli can be a bias. Virtual reality provides a possible approach to improve ecology while keeping stimulus control. We propose a new approach to study responses to FEs. A human avatar in a virtual environment (a plaza) performs the six universal FEs along the time. The setup consisted of a 3D projection system coupled with a precision-position tracker.

Subjects (N=6, mean age=25.6y) wore a 32-channel EEG cap together with 3D glasses and two infrared emitters for position tracking. The environment adapted in real time to subjects’ position, giving the feeling of immersion. Each animation was composed by the instantaneous morphing of the FE, which is maintained for one second before the ‘unmorphing’ to the neutral expression, which takes another second. Inter-trial interval was set to three seconds, keeping the neutral facial expression as baseline for one second before the morphing of any facial expression.

For the occipito-temporal region, we found a right asymmetrical negativity [150-350]ms after stimulus onset. Timefrequency analysis showed a significant difference in the beta frequency band (20-25Hz) around 350ms in the temporal lobe for the processing of the different facial expressions. This result suggests an important role played by the temporal lobe in discriminating facial expressions. Furthermore, this study provides a proof-of-concept of the possibility of using a complex virtual reality setup coupled with an EEG system for the study of dynamic and ecological social stimuli.
Spatial memory impairments in a prediabetic rat model

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Keywords: Prediabetes, hippocampus, spatial memory.

Diabetes is associated with an increased risk for brain disorders, namely cognitive impairments associated with hippocampal dysfunction underlying diabetic encephalopathy. However, the impact of a prediabetes state on cognitive function is unknown. Therefore, we now investigated whether spatial learning and memory deficits and the underlying hippocampal dysfunction were already present in a prediabetes animal model. Adult Wistar rats drinking high-sucrose (HSu) diet (35% sucrose solution during nine weeks) were compared to controls drinking water. HSu rats exhibited fasting normoglycemia accompanied by hyperinsulinemia and hypertriglyceridemia in the fed state, and insulin resistance with impaired glucose tolerance confirming them as a prediabetes rodent model. HSu rats displayed a poorer performance in hippocampal-dependent short- and long-term spatial memory performance, assessed with the modified Y-maze and Morris water maze tasks, respectively; this was accompanied by a reduction of insulin receptor-β density with normal levels of insulin receptor substrate-1 pSer636/639. Importantly, HSu animals exhibited increased hippocampal levels of AMPA and NMDA receptor subunits GluA1 and GLUN1, respectively, whereas the levels of proteins markers related to nerve terminals (synaptophysin) and oxidative stress/inflammation (HNE, RAGE, TNF-α) remained unaltered. These findings indicate that 9 weeks of sucrose consumption resulted in a metabolic condition suggestive of a prediabetic state, which translated into short- and long term spatial memory deficits accompanied by alterations in hippocampal glutamatergic neurotransmission.

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Abstracts

Pharmacology
Iron metabolism and histological changes in a rat model of chronic renal failure under high rhEPO dose therapy

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Keywords: chronic renal failure; iron metabolism; rhEPO, anemia; kidney histology

Chronic kidney disease (CKD) patients under recombinant human erythropoietin (rhEPO) therapy, usually, present an anemia associated with alterations in iron metabolism, which are enhanced in patients who develop resistance to rhEPO therapy. This study intended to evaluate iron metabolism, at biochemical and molecular levels, in a model of chronic renal failure (CRF) and of resistance to rhEPO due to formation of anti-EPO antibodies.

Three groups (n=7 each) of male Wistar rats (280 g), were studied during 12 wks: Sham: CRF: 5/6 nephrectomy; CRF+rhEPO(200): CRF treated with 200 IU/kg/wk (s.c.) of beta-EPO (Recormon®). Blood samples were collected to monitor hematological and biochemical data, including IL-6 and serum iron metabolism markers (iron, transferrin and ferritin). Liver expression of EPO, EPO receptor (EPOR) and iron related genes (by RT-qPCR) were evaluated. Anti-EPO antibodies were measured by ELISA. Kidneys lesions were analyzed with H&E and PAS staining.

CRF rats developed anemia, with a significant decrease in RBC count and Hb concentration. rhEPO(200) treatment in CRF animals corrected anemia until the 9 wks, and, afterwards the rats developed anemia due to production of anti-EPO antibodies. Furthermore, CRF animals showed a significant increase in BUN and creatinine levels, which were further aggravated in the last 3 wks; that deterioration was slightly prevented by rhEPO (200IU). Serum iron content decreased significantly in CRF rats, and rose in CRF+rhEPO(200) group. Furthermore, the CRF+rhEPO(200) group presented several significant changes in liver gene expression: increased EPOR, TRFr2, HJV, HFE and DMT1, decreased SLC40 and TMPRSS6, and a trend towards lower expression of Hamp gene (hepcidin). We also observed an overexpression of liver IL-6 mRNA. The renal impairment found in CRF rats was accompanied by kidney tubulointerstitial, glomerular and vascular lesions. rhEPO therapy aggravated the tubulointerstitial lesions.

Our study showed that the correction of anemia in CRF is closely dependent on rhEPO therapy. Since the formation of anti-EPO antibodies leads to pure red cell aplasia, triggering hypoxia, it would be expected to observe changes in iron related expression inducing an increase in iron absorption and mobilization to improve erythropoiesis; indeed, the reduction in Hamp, which is not in accordance with the changes observed in the other up-regulating hepcidin gene expression, can be attributed to the presence of high levels of EPO. Therefore, it seems that EPO immunocomplexes could have an inhibitory effect on hepcidin. Although rhEPO therapy was associated with correction of anaemia, there was a negative impact on the kidney, viewed by aggravation of tubulointerstitial lesions and this profile is related to the high dose and/or the development of resistance induced by anti-rhEPO antibodies.

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Purinergic metabolism drives a set shifting of microglial responses to different danger signals

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Microglial cells orchestrate inflammatory responses in brain disease. While beneficial at initial stages, inflammation must be time-limited, otherwise it exacerbates disease progression. Thus, controllers of microglia physiology, such as ATP and adenosine are attractive targets for disease modifying drugs. We reported recently that adenosine A2A receptor (A2AR) activation bolsters microglial proliferation, an effect requiring ATP conversion into adenosine since preventing ATP metabolism (yielding high ATP levels) impairs proliferation. This agrees with the fact that microglia surrounding damaged neurons, where ATP levels are high, do not proliferate and other cells must be recruited to phagocyte damaged neurons. We now tested if A2AR modulate the phagocytic capacity of microglia in conditions where ATP levels are low (adding the bacterial antigen, lipopolisaccharide, LPS) or high (adding glutamate). A microglial cell line (N9) was exposed for 6 h to LPS 100 ng/ml or to glutamate 0.5 mM in the absence or presence of the A2AR antagonist SCH58261 50 nM. Phagocytosis was then measured by measuring (confocal z-stack analysis) the number of internalized apoptotic cells (aged red blood cells) during 30 min. We found that neither LPS nor glutamate modified phagocytic capacity, whereas A2AR blockade inhibits phagocytosis by 20-30% (n=2). These preliminary results suggest that phagocytosis is controlled by A2AR and is independent of ATP levels. This contrasts with proliferation, which is affected by ATP levels and controlled by A2AR only in conditions of low ATP levels. Altogether, the data suggest that two key microglial responses, proliferation and phagocytosis, are differentially regulated by ATP and adenosine.

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Methamphetamine induces alterations in brain aquaporin 4 expression

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Keywords: Methamphetamine, aquaporin-4, astrocytes, hippocampus, brain edema

Methamphetamine (METH) is a potent and highly addictive psychostimulant which consumption in Europe has been increased over the last years. Several reports have demonstrated that oxidative stress, inflammation and mitochondrial dysfunction are some of the neurotoxic features of METH. Recently, it was shown that METH can also compromise the blood-brain barrier (BBB) function and cause cerebral edema. Large water fluxes continuously take place between the different compartments of the brain, as well as between the brain parenchyma and the blood. Disturbances in this well-regulated water homeostasis may have deleterious effects on brain function. Aquaporins (AQPs) are water channels that contribute to water transport across BBB. AQP4, one of the most important at the Central Nervous System, is express on astrocytic end-feet in contact with brain vessel, and can originate cerebral edema due to abnormally increased water content and consequent brain swelling. Indeed, brain edema has been observed in several neuropathologies, including under conditions of METH consumption.

Therefore, the aim of the present work was to investigate the effect of METH on AQP4 expression and brain edema. Our results show that METH increased AQP4 protein levels in primary cultures of mouse astrocytes, without interfering with the glial fibrillary acidic protein (GFAP) levels. In accordance, an acute METH treatment (4 x 10mg/kg, 2h apart) increased AQP4 expression in the hippocampus. On the other hand, there was a decrease in the frontal cortex and no alterations of striatal AQP4 levels. Regarding water content of total brain, we did not identified alterations induced by METH.

Overall, our results show that METH causes an increase in AQP4 expression in both primary cultures of astrocytes and in the mice hippocampus but without causing any alterations in total brain water content. Additionally, we also demonstrated that different brain regions present different susceptibilities to METH.

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Sitagliptin prevents inflammation and apoptotic cell death in the kidney of type 2 diabetic animals

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Keywords: Diabetic nephropathy; sitagliptin; inflammation; apoptotic cell death; type 2 diabetic model

Diabetic nephropathy, one of the major microvascular complications of type 2 diabetes mellitus (T2DM), is the leading cause of end-stage renal disease. A significant percentage of T2DM patients (20-40%) are susceptible to the development of the renal disease, requiring dialysis and/or transplantation. This pathology is characterized by excessive accumulation of extracellular matrix, thickening of glomerular basement membrane and cell hypertrophy, which ultimately progress to glomerulosclerosis and tubulointerstitial fibrosis. Inflammation and apoptotic cell death play an important role in the development and progression of renal injury. A novel class of oral antidiabetic agents, the dipeptidyl peptidase IV (DPP-IV) inhibitors, in which is included sitagliptin, has shown to improve glycemic control by stabilizing the active incretin hormones and therefore increasing insulin secretion in T2DM patients. To date, little is known about their potential benefits in the treatment of diabetic nephropathy. In this context, the aim of this study was to evaluate whether sitagliptin can exert beneficial and protective effects in an animal model of T2DM, the ZDF (fa/fa) rat.

Obese diabetic ZDF (fa/fa) at 20 wks of age were treated with sitagliptin (10 mg/kg bw/day) during 6 wks. Insulin, glucose and HbA1c were evaluated in plasma, serum and total blood, respectively. The mRNA and/or protein content of CD26, GLP-1, TNF, IL-1 β, BAX, Bcl-2 and Bid was evaluated in the kidney by RT-PCR and/or western blotting. The protein distribution was evaluated by immunohistochemistry.

At 26 wks of age, diabetic rats showed an increased inflammatory and apoptotic state. Diabetes significantly decreased GLP-1 protein levels and its immunoreactivity in the kidney. The immunoreactivity of the proinflammatory cytokines IL-1β and TNF was increased in cells around the glomeruli, probably tubular cells and/or interstitial inflammatory cells. Chronic hyperglycaemia also induced a significant increase in BAX/Bcl-2 ratio, accompanied by an increase in the levels of pro-apoptotic protein Bid, showing that diabetes increases the pro-apoptotic state in the kidney. The mRNA levels of IL-1β, TNF and BAX were also increased in the diabetic kidney, whereas Bcl-2 mRNA levels decreased. In the diabetic animals, sitagliptin treatment promoted an improvement in the glycaemic control, as reflected by a significant decrease in HbA1c levels by about 1.2%, and was able to prevent the decrease in GLP-1 levels in the kidney. Concerning the proinflammatory cytokines, sitagliptin markedly decreased the immunoreactivity and mRNA levels of IL-1β and TNF in the diabetic kidney, as well as prevented the increase of BAX/Bcl-2 ratio and Bid protein levels.

In conclusion, sitagliptin treatment has beneficial effects on the diabetic kidney in ZDF rats, possibly by a mechanism involving an improvement in renal lesions with consequent reduction in apoptosis and inflammation.

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Sitagliptin treatment prevented pancreatic lesions evolution in a rat model of type 2 diabetes – proposal of antioxidant, antiapoptotic, antiinflammatory and proproliferative mechanisms

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Keywords: diabetes; pancreas; sitagliptin; mechanisms of citoprotection; animal model

Sitagliptin belongs to a class of oral antidiabetic drugs, the gliptins, which inhibit the enzyme dipeptidyl peptidase-4 (DPP-4) that degrades incretins, thus prolonging their physiological actions. GLP-1, a prominent active compound of the incretin family, modulates many processes in pancreatic islet: it potentiates insulin synthesis and secretion, inhibits glucagon secretion, increases islet cell proliferation, and decreases cell apoptosis. In murine models of diabetes, GLP-1 has also reversed the loss of beta-cell mass by both increasing new cell formation and decreasing apoptosis. The exact mechanisms underlying the putative citoprotective effects of sitagliptin against pancreatic lesions evolution remain to be elucidated, which was the main goal of this study, using an animal model of obese type 2 diabetes (T2DM), the Zucker Diabety Fatty (ZDF) rat.

Male obese diabetic ZDF (fa/fa) rats, 20-weeks-old, were treated with vehicle or sitagliptin (10mg/kg BW/day) for 6 weeks, and compared with lean control littermates (n=8/each). Biochemical parameters and lipid peroxidation were evaluated in serum/blood/tissues. Pancreatic lesions were assessed semiquantitatively by routine histopathological and PAS staining methods. Expression in mRNA of apoptotic (Bax, Bcl2, caspase 9), inflammatory (TNFα, IL-1β, IL6), proliferative (PCNA) and angiogenic (VEGF) mediators was assessed by RT-qPCR. Immunohistochemical methods were used to confirm Bax/Bcl2 protein expression. Results are means ± s.e.m. ANOVA and Post-hoc tests were used (P<0.05 was considered statistically significant).

Sitagliptin treatment of diabetic ZDF (fa/fa) rats, ameliorated biochemical serum/blood parameters, pancreatic lipid peroxidation and diabetic lesions. An antiapoptotic effect was suggested by the reduced Bax/Bcl2 ratio, assessed both the expression of protein by immunohistochemistry and the mRNA by RT-qPCR. Caspase 9, IL-1β mRNA expression was decreased and proliferative (PCNA) and angiogenic (VEGF) factors overexpressed.

In conclusion, sitagliptin, in this animal model of T2DM, may derive its protective pancreatic effect by antioxidant, antiapoptotic, anti-inflammatory and proproliferative/proangiogenic mechanisms.

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Early cardiac changes in a rat model of prediabetes: brain natriuretic peptide overexpression seems to be the best marker

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Keywords: Brain natriuretic peptide; Diabetic cardiomyopathy; Fibrosis and Hypertrophy; High-sucrose diet; Prediabetes.

Diabetic cardiomyopathy (DCM) is viewed as a specific cardiomyopathy and defined as structural and functional changes in the myocardium due to metabolic and cellular abnormalities induced by diabetes mellitus (DM). The transition from the early metabolic abnormalities that precedes diabetes, for example impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), to diabetes may take many years; however, most individuals with these pre-diabetic states eventually develop DM. During the prediabetic state, the risk of CV events is already increased and myocardial abnormalities might appear prior to the diagnosis of Type 2 DM. Thus, the earlier identification of cardiac changes in prediabetic/insulin resistance (IR) conditions could be a better strategy to prevent the evolution to most serious stages of the disease. To elucidate whether the initial stages of cardiac dysfunction are already present in a prediabetic state with IR, and the mechanisms involved, we tested a putative animal model that might mimic a human prediabetic state of IR, without other complicating factors that could lead to cardiac events, consisting on a high sucrose (Hsu) diet during 9 weeks.

Two groups of 16-week-old Wistar rats were maintained in a 9 week protocol: high sugar (HSu: 35% of sucrose in drinking water) diet group (n=8) vs the vehicle control group (n=7). The following parameters were assessed: blood pressure, heart rate, heart and left ventricle (LV) trophism indexes and protein and/or mRNA expression of markers of fibrosis, hypertrophy, proliferation, apoptosis, angiogenesis, endothelial function, inflammation and oxidative stress. Results are mean±s.e.m. (P<0.05 was considered as significant: student’s t-test).

The HSu-treated rats presented normal fasting plasma glucose (FPG) but impaired glucose tolerance (IGT), accompanied by hyperinsulinemia and insulin resistance (P<0.01 for all), confirming this rat model as prediabetic. Furthermore, although hypertriglyceridemia (P<0.05) was observed, obesity and hypertension were absent. Concerning cardiac tissue evaluation, our results indicated that 9 weeks of treatment might be associated with initial changes, as suggested by the increased LV mass/body weight ratio (P<0.01) and remarkable brain natriuretic peptide (BNP) mRNA overexpression (P<0.01), together with a trend to upregulation of other mediators of fibrosis/hypertrophy and of angiogenesis and endothelial lesion, as well as oxidative stress (trend to increased serum and heart lipid peroxidation) and compensatory antioxidant response (increased serum total antioxidant status and cardiac overexpression of SOD, as well as downregulation of HNE and RAGE mRNA/protein levels).

In conclusion, our results suggest that 9 weeks of HSu exposure might be a short period to promote pronounced cardiac changes. However, the initial changes are already in place, deserving further elucidation.

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Pyridoxamine effects in macrovascular complications

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The present study was designed to investigate whether pyridoxamine (PM) could directly influence endothelial function, oxidative stress and inflammation in Wistar and Goto-kakizaki [(GK) rats, an animal model of type 2 diabetes], previously treated with methylglyoxal (MG).

Wistar and GK rats treated with MG in the drinking water for 3 months were treated during one month with PM and compared with the respectively control rats. The effects of PM were investigated on NO-dependent vasorelaxation in isolated rat aortic arteries from the different groups. Insulin resistance, NO bioavailability, glycation, a pro-inflammatory biomarker monocyte chemoattractant protein-1 (MCP-1) and vascular oxidative stress were also evaluated.

Methylglyoxal induced endothelial dysfunction in normal Wistar rats and aggravated the endothelial dysfunction present in GK rats. Pyridoxamine treatment significantly improved the efficacy of NO-dependent vasorelaxation. This improvement was accompanied by a decrement in the oxidative stress marker nitrotyrosine. AGEs formation was significantly decreased as well as MCP-1 and the expression of the receptor for AGEs (RAGE). NO bioavailability was significantly enhanced and accompanied by a decrement in superoxide anion immunofluorescence.

These results indicate that PM improved endothelial dysfunction in Wistar and GK rats treated with MG. The mechanism is at least in part by inhibiting oxidative stress and/or AGEs formation with a concomitant decrement of inflammation.
Kidney mTOR and Mki67 overexpression as biomarkers of transition from cyclosporine-induced renal dysfunction to nephrotoxicity

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Keywords: short and long-term cyclosporine-induced nephrotoxicity; biomarkers; gene expression; histology; rat model.

Calcineurin inhibitors, in particular Cyclosporin A (CsA), remain the cornerstone of immunosuppressive regimens in many transplantation centres worldwide, regardless of drug-induced nephrotoxicity. The pathogenesis of CsA-induced nephropathy remains to be fully elucidated, but it seems to be affected by the duration of drug exposure. This study aimed to clarify the pathways involved in short and long-term CsA-induced nephrotoxicity.

The study comprised 24 male Wistar rats, divided in two models: acute and chronic (3 and 9 weeks of treatments, respectively). Each model included two rat groups (n=6 each), receiving orally: Control group – vehicle; CsA group – 5 mg/Kg BW/day. Blood pressure and heart rate were monitored. Blood was collected at 0, 3 and 9 weeks to evaluate CsA concentrations, biochemical parameters, including lipid profile measures, glycemia and insulin, as well as hematologic data, including lymphocyte populations. Renal function was assessed on serum, urine and kidney tissue samples, through creatinine, BUN and TBARs measures, including clearances. Renal tissue was also used to gene expression (qRT-PCR) analysis of markers of inflammation (IL-1β, CRP, TNF-α, NF-κβ, COX-1 and COX-2), proliferation (TGF-β1, PCNA, Ki67, mTOR and TP53) and angiogenesis (VEGF) was performed. mTOR protein levels in the kidney tissue were assessed by immunohistochemistry. Hematoxilin & eosin, periodic acid of schiff and masson’s trichrome staining were used to evaluate glomerular, tubular and vascular kidney lesions.

CsA has promoted hypertension and tachycardia, which were aggravated with the duration of exposure. Creatinine and BUN clearance and GFR showed early renal dysfunction, accompanied by increase serum creatinine (p<0.05) and BUN (p<0.01) levels, as well as kidney lipid peroxidation (p<0.05), which worsened with long-term exposure. Renal lesions were evident only after the 9 weeks treatment period. However, short-term CsA exposure induced PCNA and TGF-β1 kidney mRNA up-regulation (p<0.05), unchanged mTOR and down-regulation of Mki67, while chronic treatment revealed a normalized PCNA and TGF-β1 expression, accompanied by prominent mTOR and Mki67 up-regulation (p<0.01).

In conclusion, different molecular biomarkers distinguishes short and long-term CsA-induced nephrotoxicity, being mTOR one of the key players for kidney lesion evolution, suggesting early CsA substitution by mTOR inhibitor as a feasible therapeutic choice.

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Abstracts

Other
Synthesis optimisation of [68Ga]DOTA-NOC for routine GMP production

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Keywords: Gallium-68; DOTA-NOC; GMP; Radiolabelling

In recent years, Gallium-68 labelled peptides, such as [68Ga]DOTA-NOC, have become established tools for the evaluation of SSTR-expressing tumours with PET. Therefore, a simple and reproducible process for labelling peptides with generator-produced 68Ga under GMP conditions is of great importance. We describe the development and validation of a cassette-based method for 68Garadiolabelling of peptides using an automated commercial module (Synthera®, IBA, Louvain-la-Neuve, Belgium) coupled with a standard TiO2-based 68Ge/68Ga generator (Eckert & Ziegler, Berlin, Germany). A complete method using Ph. Eur. reagents and a robust synthesis sequence was developed and implemented. The 68Ge/68Ga generator is slowly eluted with 5mL of 0.1M HCl directly to a cation exchange column (Bond Elut-SCX 100mg, Agilent Technologies, USA) to retain the 68Ga. Other impurities, such as any eventual 68Ge, pass through the column without being trapped. The column is then dried with a stream of inert gas. 68Ga is eluted with 600 µL of a 98% acetone/0.02M HCl solution into a reactor containing 30µg of the DOTA-NOC peptide in 1mL of a HEPES 0.5M buffer. The reaction mixture is then heated for 15 minutes at 105ºC. After the reaction, the mixture is transferred to a preconditioned OASIS HLB light C18 purification cartridge (Waters, Massachusetts, USA) and washed with 10ml of deionized water. 68GaDOTA-NOC is then eluted from the C18 with 1mL of EtOH and 9mL of physiological saline followed by sterile filtration via a 0.22µm Millex®GV (Millipore, Billerica, USA) filter. The final product is tested for chemical and radiochemical purity, pH and residual solvents. [68Ga]DOTA-NOC was successfully synthesized in about 25 minutes with a radiochemical yield of 70.7±11.4 % (DC). Labelling efficiency was 83.47±10.25%, and the purification, formulation and sterile filtration were achieved with >98% yields using the C18 cartridge eluted with 1mL of EtOH. The RCP of the final labelled product was better than 99%, pH was between 5-7 and by GC we found that there were no residual solvents apart from ethanol (<10% of final concentration). Sterility (tested by an independent laboratory) and a pyrogenicity (gel-clot) tests were all negative. All results were within the specification of the Ph. Eur. for 68Ga-labelled peptides. Validation data was submitted to the Portuguese Licencing authorities and a Manufacturing Authorization was granted in June 2013. An automated process for [68Ga]DOTA-NOC production using a commercial synthesiser was successfully developed for a reliable and reproducible routine production. The RCY of the final product are reproducible, stable and show excellent quality with high RCP and RNP. With this system and dedicated disposable cassettes, the synthesis is safe and suitable for a GMP production process. The process can easily be adapted for the labelling of other peptides and other radiometals.
INFLUENCE OF THE LIGATION METHOD, METALLIC ALLOY AND TIPPING IN SLIDING MECHANICS

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KEY WORDS: Sliding Mechanics, Self-ligating brackets, Friction, Resistance

INTRODUCTION: Since the development of orthodontic fixed appliances, bracket design has undergone many modifications in order to improve treatment efficiency. In the last decades, the popularity of self-ligating brackets has grown based on manufacturers claims of lower friction, faster ligation, less chair time, fewer appointments, shorter treatment time, increased comfort and less pain. The resistance to bracket sliding is a main factor influencing treatment time and eventually the outcome of the orthodontic treatment, therefore is important to ascertain how different bracket prescriptions respond to friction. The aim of this study was to evaluate, in vitro, the resistance in sliding mechanics of conventional ligated brackets and of both active and passive self-ligating brackets when using stainless steel and nickel-titanium archwires; and the effect of tipping on the resistance to such sliding mechanics.

MATERIAL AND METHODS: The 0.022-inch slot brackets Damon® Q™; Prodigy SL™; Smart-Clip™SL3; Victory Series™; Morelli® Roth Standard and Morelli® Roth SLI were tested. The brackets were ligated to 0.016 x 0.022 inch stainless steel and nickel-titanium archwires. A tipping of 0° or 5° was added to the wires. For each combination of bracket/archwire, 10 sliding tests were performed with the Shimadzu AG-1 5kN testing instrument.

RESULTS: A higher resistance to the sliding was observed with the conventional ligated brackets when compared with the self-ligating brackets, passive or active. There were no statistical differences either between the resistance of active and passive self-ligating brackets or between the resistance of both types of orthodontic archwires for an angulation of 0°. The stainless steel archwires demonstrated a higher resistance for an angulation of 5°. There were no statistical differences between 0° and 5° of tipping.

CONCLUSION: The self-ligating brackets are an useful tool in orthodontic mechanics, when low levels of friction are needed. When used with small diameter archwires, the resistance to sliding is not affected by small angulations and small degrees tipping. Nevertheless, different metallic alloys present dissimilar behaviour when angulations are present.
#P93

**Development of insulin delivery systems: micro-encapsulation of islets of Langerhans with polymeric materials**

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**Keywords:** Diabetes; Islets of Langerhans microencapsulation; Alginate

Due to the increasing incidence of diabetes, the high rates of pancreatic cancer and other metabolic diseases that compromise the beta cell functioning, it is urgent to develop a reliable and safe source of insulin production. The purpose of islet microencapsulation technique is to allow the islet transplantation in the absence of immunossupression by protecting the cells from the host through an immunoisolative membrane that enables adequate transport of oxygen, nutrients, and secreted hormones. However, the in vivo viability of this kind of devices has been shown to be compromised. Thus, the objective of this work is to understand and characterize the optimal conditions to maintain functional islets in vivo and to develop a device that meets these characteristics. Accordingly, we selected different bio-substances that are crucial to protect encapsulated cells and improve their functioning: anti-ROS, anti-inflammatory and insulinogetic agents and also a pro-angiogenic agent. Regarding to the polymers, we are using alginate, a natural polymer commonly used in islets microencapsulation, which, with the specific modifications that we have done, will be able to interact with those substances. Aiming to use fluorescent microscopy to observe the coatings we prepared fluorescent alginate. For further reactions with amine and acrylate groups, oxidized alginate and functionalized oxidized alginate with 2-aminoethyl methacrylate were prepared, respectively. The synthesized polymers were characterized by FTIR and NMR. Furthermore, with the objective to reduce the size of the particles as much as possible, we are also testing different approaches to encapsulate the islets.
Characterization of the macrophage response to Alternaria infectoria conidia infection

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Keywords: Alternaria infectoria, conidia, macrophage, immune response

Alternaria infectoria is an environmental mould responsible for phaeohyphomycosis in immunocompromised hosts. The major prevalence of airborne Alternaria spores can lead to opportunistic fungal infections. Additionally, the increasing number of reports describing clinical manifestations of allergies caused by these moulds further supports its significance in human health. A. infectoria spores recognition and elimination through host phagocytic cells, namely macrophages, is an essential step towards pathogen clearance. Macrophages are also key players in the generation of an antifungal innate immune response, both through promotion of phagocytes migration and induction of proinflammatory cytokines and chemokines.

In this work we investigated the interaction between RAW 264.7 macrophage cell line and A. infectoria spores. We found that no major proinflammatory response was triggered after co-culture of RAW 264.7 macrophages with A. infectoria spores; the levels of TNF-α, a proinflammatory cytokine, did not increase significantly; no substantial increase of extracellular ATP (an endogenous danger signal) was observed; and, no classical associated morphology changes were found in infected macrophages. Moreover, live cell imaging evidenced that A. infectoria spores are rapidly internalized by macrophages and those phagocytosed spores are able to germinate intracellularly. Also, macrophages retain the ability to divide by mitosis while containing internalized spores. Subsequently, we explored if the adenosine system (through activation of A2A adenosine receptor, A2AR), was involved in the low activation of macrophages. Although A2AR were found to accumulate in clusters during A. infectoria spores infection, the levels of Adora 2a gene expression remained unchanged in macrophages infected with A. infectoria spores.

Overall, the present results show that RAW 264.7 macrophages are unable to generate an effective antifungal response against A. infectoria spores, suggesting that this opportunistic fungal pathogen may employ latency-like mechanisms in order to hamper macrophage function.
**#P95**

**Effect of cell wall synthases inhibitors in the modulation of cell wall chitin and glucan synthesis of Alternaria infectoria**

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**Keywords:** Alternaria infectoria, caspofungin, nikkomycin Z, chitin, glucan

Alternaria sp. are filamentous fungi with cell walls containing melanin species that are increasingly found as agents of human allergies and as opportunistic agents of infections. Before, we identified Alternaria infectoria as an agent of a cerebral abscess (Hipólito et al., 2008) and characterised A. infectoria genes that code for one of the cell wall components (Anjos et al., 2012). Like other fungi, these are provided with a shielding cell wall made of interconnected chitin, glucans, mannans and glycoproteins. Chitin and glucan are the perfect targets for antifungal drugs because they are essential to fungal cell growth and to survival against environmental deleterious conditions. Two classes of antifungal drugs directed to these cell wall components were developed, the echinocandins, such as caspofungin, non-competitive inhibitors of the β-1,3-glucan synthase and the chitin synthases inhibitors, that includes nikkomycin Z.

The present communication reports the effect of caspofungin and nikkomycin Z in two strains of A. infectoria. We identified eight different chitin synthase genes and studied the relative gene expression when the fungus was exposed to the antifungals under study. We report that one A. infectoria strain bears phenotypic traits that provide it some advantages to stand with antifungal treatment. First, the basal chitin cell wall content is higher in this strain, IMF006 and, in response to the β-1,3-glucan synthase inhibitor, caspofungin, its cell wall chitin level remained constant while in the other strain, IMF001, the chitin content increases to values similar to basal IMF006 levels. More, upon caspofungin treatment the FKS gene is up-regulated in IMF006 and down-regulated in IMF001. On the other hand, the β-glucan content is also different in both strains, with higher levels in IMF001 than in IMF006 and does not provides any advantage towards antifungal resistance. Caspofungin does not change the pattern of A. infectoria spores phagocytosis, indicating that the β-glucan at surface is not changed by this antifungal. While separately the antifungals only provide a fungistatic effect, the combination of both lead to fungal cell lysis, revealing a strong synergy between chitin synthase inhibitors and β-glucan synthetase inhibitors.

In conclusion, the chitin level determines the susceptibility to anti-cell wall antifungals.


Portuguese Dentists’ role in addressing obesity

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Keywords: Obesity; survey; questionnaire; translation; validation.

Aim or purpose: overweight and obesity is a major public health in Portugal. There are various factors contributing to this disease and dentists can incorporate into their patient encounters interventions to this problem. The aim of this work is to know the Portuguese dentists’ role in addressing obesity.

Materials and methods: to translate from English into Portuguese (and back-translation) the original version of Dentists’ role in addressing obesity. Provide cultural adaptation (validation by an expert committee) for Portuguese dentists. Apply the questionnaire to a random sample of 400 Portuguese dentists and perform a statistical analysis.

Results: in all, 141 dentists responded. Overall, 22.0 percent of respondents offered a form of counseling services and 58.9 percent reported that they were interested in offering obesity-related services. A paucity of trained personnel (58.9 percent) was cited by the respondents as a major barrier, followed by patients’ rejection of weight-loss advice (32.6 percent) and fears of offending patients (29.1 percent). Ninety-two percent of respondents agreed that dentists would be more willing to intervene if obesity was linked to oral disease.

Conclusions: health care providers must coordinate prevention and interventional efforts for maximum effect.
Given the positioning of dentists willing to assist in such an effort, it appears reasonable for experts in obesity intervention in conjunction with dental educators, to develop intervention models to be implemented within the scope of dental practice.
#P97

The effect of bleaching agents on the surface roughness and microhardness of composites

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Keywords: tooth bleaching/ resin composite/ roughness/ microhardness

**Introduction:** Currently dentistry is experiencing a trend of increasing demand from patients for superior aesthetic restorations. Very often in daily clinical practice, tooth colored restorations exist in teeth that are planned to be bleached. Therefore, it is important to understand the effects of bleaching agents on the physical properties of the restorative materials.

**Methods and materials:** Sixty cylindrical specimens (10mmx2mm) of each composite were prepared, polished and divided into 6 groups (n=20). Groups 1, 2: stored in artificial saliva. Groups 3, 4: 10% carbamide peroxide. Groups 5, 6: 35% hydrogen peroxide with LED lamp activation. 24 hours after treatments, specimens went through 500 cycles of thermocycling between 5°C and 55°C with a dwell time of 30 seconds.

A mechanical roughness tester was employed to measure the surface roughness parameters and the Vickers test to measure microhardness on the top surface of each specimen. One-Way-ANOVA, Tukey and Bonferroni methods with a significance level of 5% were used for the statistical analysis.

**Results:** There was no statistically significant difference in microhardness between the control groups (1, 2) and the bleached groups (3, 4, 5, 6). However there was difference between home bleaching (group 3) and in-office treatment (group 5).

In case of roughness, there was no significant difference in roughness average (Ra) and root mean square roughness (Rq) among all groups. The mean roughness depth (Rz) parameter showed no statistically significant differences among all groups for SonicFill™ but, in Filtek Supreme XTE™ there was a significant increase between control (group 2) and bleaching treatments (groups 4, 6).

Roughness skewness (Rsk) showed no statistically significant differences among all groups for SonicFill™ and Filtek Supreme XTE™, except for groups 2 and 4, where the Rsk increased with CP.

**Conclusions:** The microhardness of Filtek Supreme XTE™ and SonicFill™ is not affected by bleaching treatments.

Both bleaching treatments affect Rz of Filtek Supreme XTE™ groups, in contrast to the SonicFill™ groups.

The carbamide peroxide 10% treatment affects the Rsk of group Filtek Supreme XTE™ with no significant effect in the SonicFill™ group.
ORTHODONTIC TOOTH MOVEMENT – A BIOLOGICAL STUDY

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Keywords: Orthodontic tooth movement, Wistar rats, Periodontal Ligament

Introduction – Orthodontic treatment always need a careful biological and mechanical consideration, mostly to prevent side effects as root resorption and other periodontal problems that can lead to irreversible bone destruction. When an orthodontic force is applied to the teeth, their surrounding periodontal tissues respond with a series of biological reactions, which result in the remodeling of the alveolar bone and periodontal ligament and subsequently, orthodontic tooth movement is provoked. In orthodontics, the application of a light and continuous force is recommended, since under low force magnitude, cellular activity of the periodontal ligament occurs, promoting a physiologic and steady tooth movement.

Material and methods – 35 Wistar rats, aged 12 weeks were used in the study. The right and left maxillary first molars were moved mesially by the Waldo method. The wistar rats were sacrificed at 24 hours, 48 hours and 72 hours after orthodontic force appliance. The tissue was collected and prepared to perform the histological analysis.

Results - At 24 hours, periodontal ligament organization; inflammation; and alveolar bone resorption were detected. At 48 hours, is possible to observe enlargement of the periodontal space and remodeling of alveolar bone with apposition of osteoid at the pressure side. At 72 hours, the periodontal ligament appears to be hypercellular; being also present alveolar bone remodelling in the tension side.

Discussion and Conclusion – The findings of this study agree in general with those described in the literature, nevertheless, histological changes such as alteration of periodontal fibers orientation are not detected. Not all the animals have the same histological alterations. In those with the malpighian epithelium surrounding the tooth, no orthodontic movement is observed.
#P99

**Computerized occlusal analysis using T-Scan®III HD (in vitro study)**

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**Key-words:** computerized occlusal analysis; T-Scan®III system; HD sensor design; dental occlusion.

**Introduction:** The performance of registration materials and methods has been researched by many investigators in an effort to thoroughly understand the patient’s occlusion. In the dental community, articulating paper has been widely accepted as the gold standard for occlusal analysis. However, published studies about its physical properties (thickness, composition, ink substrate, plastic deformation) offer no evidence to suggest that variable articulating paper mark size can be descriptive of variable occlusal loads. In 1987, Maness et al\(^{24}\) first reported the development of the prototype of a new computerized occlusal analysis device (T-Scan® Tekscan Inc., Boston, USA). From then to the present, the manufacturer states having improved the system’s performances. The present study aims to test, under different simulated anatomic circumstances the sensitivity and reproducibility of the new T-Scan®III HD.

**Materials & Methods:** Four different occlusal tables were created:
- two of 120° created with an artificial inferior first molar (Ivoclar® Vivadent, Vaduz, Liechtenstein) either embedded in a periodontal ligament simulator (Affinis® Putty soft, Coltène/Whaledent, Aldstätten, Switzerland) or not (representing the anatomy of posterior natural teeth vs an implant);
- one of 100° (simulating the distortion created to the sensor when anterior teeth occlude);
- and finally one plane surface of 180° (control) in static and variable positions.

Three levels of force (10N, 50N and 150N) were applied, 40 times each, by a universal testing machine (Autograph®, AG-I; Shimadzu Co., Kyoto, Japan). A polished spherical bur (diameter=2,2mm) assured the contact on the sensor film. All T-Scan®III HD recordings were compared through a One-way ANOVA statistical analysis with post-hoc tests using Bonferroni corrections for multiple comparisons.

**Results:** According to our study, the following results were obtained:
- 85% of the outliers are within the 5 first closures, representing the conditioning time required by the initially flat sensor
- Graphically and statistically sustained differences (p < .05) could be found in the coefficients of variation between tables (180° Variable vs. all other)

Conclusions: An undeniable improvement of this newest T-Scan® system as compared to former designs could be proved. However, when using the T-Scan®III HD system, some points of capital importance have to be considered:
- Its sensitivity seems to be improved as compared to former designs, however further studies on its variability throughout its sensing surface are required;
- Its reproducibility could be proved. The 5 first values (outliers to the mean values) shall be used as a conditioning time to both the sensor and the patient.

Our study shows that despite the technologic advances made in the area of occlusal analysis, a trained handling of the depicted values acquired through a long learning curve is indispensable.
Exogenous adiponectin administered through a minipump reverts high-fat diet-induced impairment of adipose tissue metabolism in Wistar rats

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Keywords: Adipose tissue; adiponectin, glucose metabolism; lipid storage

It is now generally accepted that adiponectin is an essential adipose-derived hormone, which increase lipid metabolism and decrease lipotoxicity and insulin resistance in adipose tissue, muscle and liver. However, adiponectin administration to obese or type 2 diabetic patients is still a myth, due to its expensive costs and absence of studies demonstrating the effectiveness of exogenous adiponectin. Thus, our objective was to produce adiponectin in vitro and test the usefulness of this synthetic peptide in improving the metabolic profile and adipose tissue metabolism in high-fat diet fed rats.

Normal Wistar rats were fed a high-fat diet in order to induce hyperlipidemia and adipose tissue mass gain. Adiponectin (2.7 mg) was administered during one month through a subcutaneous minipump with continued release. We evaluated the metabolic profile (glucose and lipids) and assessed the activation of pathways involved in controlling lipid storage and insulin action in epididymal and subcutaneous adipose tissue.

Exogenous adiponectin reverted high-fat diet-induced increased body weight. More, adiponectin was also able to decrease fasting glycemia and cholesterol levels (total and non-HDL), even if high-fat diet did not produce a significant increase in relation to control rats. In adipose tissue, high-fat diet caused an increase of Akt activation and a decrease of IRS-1 activation, a marker of insulin resistance and hyperinsulinemia (increased insulin signaling through non-canonical pathways). This was mainly observed in epididymal adipose tissue and was consistent with decreased IkappaBalpha levels, despite no major alterations were observed in total macrophage levels in adipose tissue (F4/80). These effects were reverted by exogenous adiponectin. Regarding lipid storage, high-fat fed rats showed decreased PPARgamma levels in relation to control rats in both fat depots, which were reverted by adiponectin treatment. Consistent with hyperinsulinemia, high-fat diet fed rats showed little activation of lipolysis during fasting in epididymal adipose tissue, despite no alterations were observed in the total amount of the protein. This was partially reverted by adiponectin.

Exogenous adiponectin administered through a subcutaneous minipump was able to improve pathways of insulin signaling and lipid storage in adipose tissue after the consumption of a high-fat diet. Adiponectin was also able to improve the metabolic prolife, probably as a result of improved adipose tissue metabolism.
ROLE OF DENTISTRY IN THE OSTEONECROSIS OF THE JAW ASSOCIATED WITH BISPHOSPHONATES

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Keywords: Biphosphonates; Oral surgeries; Osteonecrosis; Jaw.

Introduction:
Osteonecrosis of the jaws is an adverse effect of bisphosphonate therapy widely used for the treatment of bone metastasis. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) can be predicted with a conjunction of genetic and environmental risk factors but still do not have an effective treatment.

Aim:
Confirm the impact of nitrogen-containing bisphosphonates (N-BP), in two different oral surgeries, and study different materials that could be applied in the oral wounds, favouring cicatrization.

Methods:
In chemistry laboratory, it was studied the reaction of zoledronate (ZOL), the most potent and widely used N-BP, with different compounds, in order to find if can efficiently bind ZOL. The cells used to test the compounds were primary cultures of gingival fibroblasts, cultured following a specific protocol. In the animal model, there were used Wistar rats and were organised into two groups, in order to compare the cicatrization of oral wounds after the extraction or periapical surgery of first inferior left molar, in a group with and in another without ZOL weekly subcutaneous administration. ZOL was labelled with Technetium-99m to image which skeleton zones that uptake more ZOL, highlighting where bone turnover was more active. Radiopgrafs were also performed and analysed using ImageJ software and histology study was performed.

Results:
Bone analysis through X-ray images show bone defect in the two groups and histological study demonstrate differences in deposition of new bone, necrotic bone and inflammatory infiltrate. Bone healing, re-epithelisation, organization of connective tissue and new osteogenesis were favoured in control group. These results were confirmed through nuclear medicine after 99mTc-Zoledronate in which the uptake coefficients were calculated after ROIs drawing.

Conclusion:
The materials tested in chemistry laboratory decreased ZOL toxicity when applied to the cells. In animal model, healing was lower in experimental versus control group. Once there is no known effective resolution for BRONJ, patients with N-BP treatment should have a preventive oral healthcare combined with nonsurgical dental procedures to reduce the need of bone manipulation.
#P102

**Beneficial effects of berberine on endothelial function in type 2 diabetic and obese animal models**

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The present study was carried out to investigate the effects of berberine on endothelial dysfunction and metabolic profile in diet induced obesity (DIO) and type 2 diabetic animal models. Sprague Dawley (SD), SD fed with high fat for 16 weeks (DIO rats), Wistar and Goto-Kakizaki (GK) rats treated with berberine in the drinking water (100 mg/kg) were compared with the respectively control rats.

DIO and GK rats showed significantly reduced endothelial function with maximal relaxation in response to acetylcholine significantly reduced to 38 and 50 %, respectively. This impairment was accompanied by an increase in superoxide anion immunofluorescence in both DIO rats and GK rats when compared with respective control rats. The efficacy of NO-dependent vasorelaxation was significantly improved with berberine while phenylephrine contraction was significantly decreased. DIO rats show a profile with metabolic syndrome characteristics. GK rats showed significantly increased fasting glycemia, HbA1c and total cholesterol. Berberine treatment significantly improved glycemic profile, vascular oxidative stress and restored endothelial function in DIO rats and GK rats.

In summary, the present results showed that berberine normalized endothelium-dependent relaxation in DIO rats and ameliorated this parameter in GK rats. The present study provides further evidence for berberine as one of the potential useful agents in the treatment of macrovascular complications associated with diabetes and metabolic syndrome.
WiSTAR RATS’ MANDIBLE DEVELOPMENT AFTER EXPOSURE TO HEXAVALENT CHROMIUM IN DRINKING WATER

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Not only workers employed at industrial plants are exposed to intoxication with the element they manipulate, the population at large is also at risk of suffering health problems caused by contaminating wastes inadequately treated for their safe disposal. As a result certain toxic substances, such as hexavalent chromium, have reached the general population. The present study sought to evaluate the effect of intoxication with hexavalent chromium on mandible and teeth in Wistar rats.

We conducted study within the Wistar rats, where water contaminated with 20ppm of hexavalent chromium as been administrated to Wistar rats mate couples and to offspring’s till the age of 20 days. At the end of the 20 days they were sacrificed, the mandibles were collected and studied by optical microscopy.

No histological changes were observed in the control group. For the opposite in the animals, of the experimental group we observed that the bone areas were not well demarked, and some lost in the disposal of the chondrocytes and accumulation of extracellular matrix. Additionally changes in the formation and density of bone were observed as delayed in the formation of the secondary centers of ossification putting in evidence the presence of cells of cartilage not reabsobered.

We can conclude that hexavalent Chromium in water although in a small dose as 20ppm can have effects on juveniles’ mandible growth and ossification.
Yeasts in foods: a new threat to an ageing population?

Rodrigues L (1,2), Curado F (1), Mota M (1,2), Coelho C (1,2), Cabral V (1), Cortes L (1), Cunha R (1,2), Gonçalves T (1,2)

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Keywords: Yeasts; Macrophage; Infection; Adenosine; Phagosome

Yeasts are important agents of severe infections, albeit being part of human body normal flora and ubiquitous in food environments. Their recognition as pathogens and clearance by host phagocytic cells is a complex procedure involving multiple systems and responses. In individuals with immune diseases or weakened immune system (as occurs in immunosenescence, the normal human ageing), a decreased ability to control infections is found. Purines, namely adenosine, are endogenous signalling molecules contributing to the fine-tuning of inflammatory and immune responses, controlling to minimal the inflicted damage.

This project, with a board goal of clarifying if different species of yeasts (Candida albicans, Candida parapsilosis, Candida glabrata and Saccharomyces cerevisiae) in diet can constitute a real threat to elder individuals, was initially divided in 4 different tasks, which after selection of clinical and food yeast isolates (task 1), was aimed to study the adhesion to non-phagocytic cells and phagocytosis (task 2), differential yeast pathogenicity, in vitro and in vivo (task 3) and the role of adenosine receptors, specially A$_{2A}$, in the efficiency of phagolysosome towards yeast clearance (task 4). Using several approaches it was elucidated the interaction between C. albicans, C. glabrata, C. parapsilosis and S. cerevisiae, with RAW 264.7 macrophage cell line and mouse primary peritoneal macrophages. In particular, it was observed that during C. albicans infection, adenosine A$_{2A}$ receptors are activated and localize around phagosomes containing yeasts cells, although the gene expression does not increases. Extracellular ATP, NTPDases activity and cytokine levels are the other related aspects explored in an attempt to understand how yeasts overcome phagocytosis. Deciphering the A$_{2A}$ trafficking to the phagosomal membrane and the immuno-inflammatory response of aged cells, as well as the matching in vivo mouse infection model to the presence of yeast cells, are part of the ongoing work.

#P105

**Effect of caffeine administration in the electrophysiological response of the retina in a model of retinal ischemia-reperfusion**

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**Keywords:** Adenosine, caffeine, electroretinography, ischemia-reperfusion, retina.

Caffeine is one of the main biologically active compounds of coffee and the most widely consumed psychoactive substance in the world. Caffeine at low concentrations can block all four subtypes of adenosine receptors (A1, A2A, A2B and A3), most actions being mediated through inhibition of the high-affinity A1 and A2A receptors.

Ocular ischemia plays an important role in the pathophysiology of various ocular diseases, which may lead to neuronal death. Retinal neuroprotection is often quantified as molecular and histological changes. However, these changes provide no retinal functional information unlike retinal electrophysiology, a non-invasive in vivo approach.

Here the aim was to investigate if long-term caffeine administration prevents changes in electroretinography (ERG) induced by retinal ischemia-reperfusion (I-R) injury.

Caffeine (1g/L) was administered in drinking water from 2 weeks prior retinal I-R injury. Intraocular pressure (IOP) was measured regularly. Caffeine did not affect IOP values when compared to control animals (water only). ERGs were recorded after dark adaptation at 4 time points: before caffeine administration; after 2wks of caffeine; 24h after I-R injury and 7d after I-R. The ERG consisted of a 4-step exam: scotopic; light adaptation; photopic and flicker.

ERG analysis of all ischemic eyes revealed a similar pattern. Scotopic condition showed severe amplitude reduction of a- and b-waves 24h and 7d post-I-R, while OP amplitudes were barely measurable. Photopic and flicker conditions also showed serious defects in amplitude of b-waves and 1\(^{st}\) harmonic, respectively. None of these ERG components showed a significant recovery at 7d after I-R injury.

This data suggests that 1g/L caffeine, given orally, did not prevent ERG alterations induced by I-R, indicating that caffeine does not protect retinal function after I-R injury.

#P106

EFFECTS OF RHYTHM OF DISTRACTION OSTEOGENESIS ON SAGITTAL MANDIBULAR LENGTHENING

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Keywords: Distraction osteogenesis; Mandible; DEXA scan

Objective: Frequency of activation during distraction can theoretically influence the process of distraction osteogenesis. The aim of the study is to evaluate the effect of two different frequencies of distraction in the amount and architecture of new bone using a tooth-borne distractor.

Materials and Methods: Ten beagle dogs, weighing between 15-18kg, were used. Three remained as the control group and seven underwent a mandibular distraction protocol. Both hemimandibles were used for experimental purposes, with the following division: Group A: Six did not undergo any surgical procedure, remaining as a control group; Group B: Seven were subjected to two daily activations of 0.5 mm, with an interval of twelve hours; Group C: Seven received a single daily distraction of 1mm. After the distraction period, all devices were properly blocked and submitted to a consolidation period of 12 weeks. The mean distraction achieved was 9.8 mm. The evaluation of bone tissue was made radiographically and by Dual X-ray absorptiometry, and the values obtained were subsequently sent for statistical analysis.

Results: Radiographic evaluation showed that a greater the consolidation period leads to a greater amount of bone tissue in the distraction gap. There were no statistically significant differences in bone mineral content and bone mineral density among groups A, B and C. There were statistically significant differences between the coefficient of variation in groups B and C (p=0.041).

Conclusions: An increased in rhythm from one to two daily activations changed the quality of new bone present in the area created by distraction.
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<td>Vale F</td>
<td><a href="mailto:franciscovale@gmail.com">franciscovale@gmail.com</a></td>
<td>EFFECTS OF RHYTHM OF DISTRACTION OSTEOGENESIS ON SAGITTAL MANDIBULAR LENGTHENING</td>
<td>Other</td>
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<td>P59</td>
<td>Vale Pereira S</td>
<td><a href="mailto:spereira@fmed.uc.pt">spereira@fmed.uc.pt</a></td>
<td>ASTHMA AND RHINOSINUSITIS: DOES GENETIC PROFILE IDENTIFIES WHO IS GETTING WHICH?</td>
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<td>P36</td>
<td>Vieira L</td>
<td><a href="mailto:luisgsvieira.tv@gmail.com">luisgsvieira.tv@gmail.com</a></td>
<td>Epigenetic modifications in Hepatocellular carcinoma - Can epigenetic modulating drugs play a role on HCC therapy?</td>
<td>Cancer</td>
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Thank you for your participation

The PostDoc Forum is a platform for postdoctoral scientists to influence matters important to them.

These include organizing activities such as workshops and symposiums that aim at bridging the gap between students and principal investigators and provide everyone with new insight and inspiration; facilitating collaboration and professional growth.

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