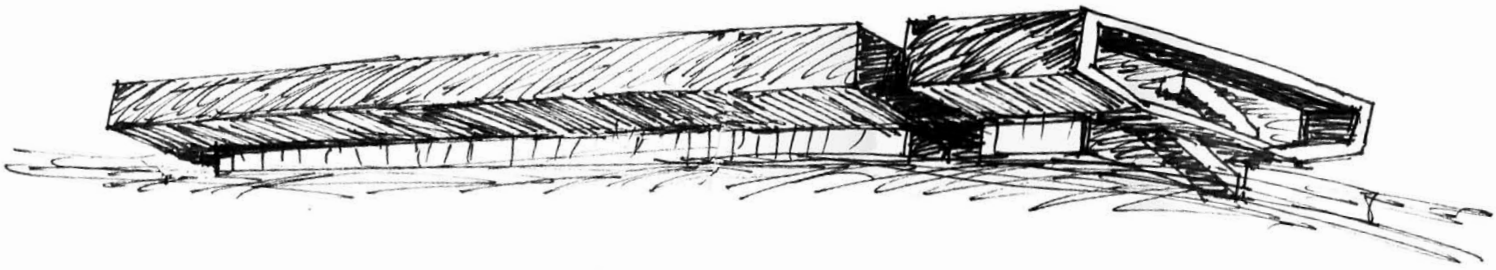
2014

4TH ANNUAL MEETING

IBMC | INEB | IPATIMUP

30-31 OCTOBER 2014
Axis Vermar
Conference & Beach Hotel
Póvoa de Varzim



ABSTRACT BOOK

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Organizing Committee

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Welcome Address

The 4th I3S Annual Meeting takes place this year on the 30-31 of October at the Hotel Axis Vermar, Póvoa do Varzim.
This meeting which is now a regular event of the new Institute, this year is specially designed to mark the advanced stage in the development of the I3S project both in terms of the construction of the new building and also the implementation of a new scientific structure that will underlay a significant part of our collective future.
The meeting will consist of three main sessions devoted to scientific talks on the topics of the I3S research programs: Host Interaction and Response; Neurobiology and Neurologic Disorders; and Cancer. Each session will include lectures selected from significant work recently published by I3S researchers, as well as additional invited speakers. We will also have a long session dedicated to accommodate the large number of posters already submitted and to foster interaction and discussions. The poster session will be followed by a information session on the I3S project, the recent site visit carried out by the review panel from FCT, the advancement in the building and other relevant information.
The main objective of the meeting is to promote scientific exchange amongst I3S collaborators and also to continue to foster closer integration on our route towards the full implementation of the project, which will occur next year.
So far we have had a very enthusiastic reception to the meeting with more than 500 participants registered and over 200 posters submitted which will certainly make this once more a great event.
We are in debt to all those who have made this event possible and hope you will have a very fruitful meeting.

The Organizing Committee

Important Information

Registration desk
The Registration desk will be open at 8:15 on October 30 and at 8:30 on October 31.

Name badges
For identification and security purposes, participants must wear their name badges when in the venue. The use of the badge is mandatory for the access to the coffee breaks, lunches and dinner.
The distribution of badges will be done in alphabetical order by first name.

Presentation instructions
The Plenary Lectures should last up to 50 minutes followed by 10 minutes of discussion. Oral presentations should last up to 20 minutes followed by 10 minutes of discussion. Chairs are asked to lead the discussion and to ensure that the times are strictly followed.
Speakers presenting in the morning of October 30 should hand in their presentations in the auditorium at 8:30. Those having their presentations in the morning session of October 31 should hand in the presentation in the auditorium at 9:00. Speakers presenting in the afternoon sessions, should hand in their presentations during lunch break.
A data show and personal computer will be at the presenters' disposal. Technicians will be available to make sure that you have successfully uploaded your presentation. You will be requested to provide your presentation in a USB key. If strictly necessary, you may use your own computer, which should be installed and properly tested prior to your presentation at the indicated times.

Poster presentations
Posters should have 1.20m high and 0.90m wide, and will be displayed on the designated poster areas:

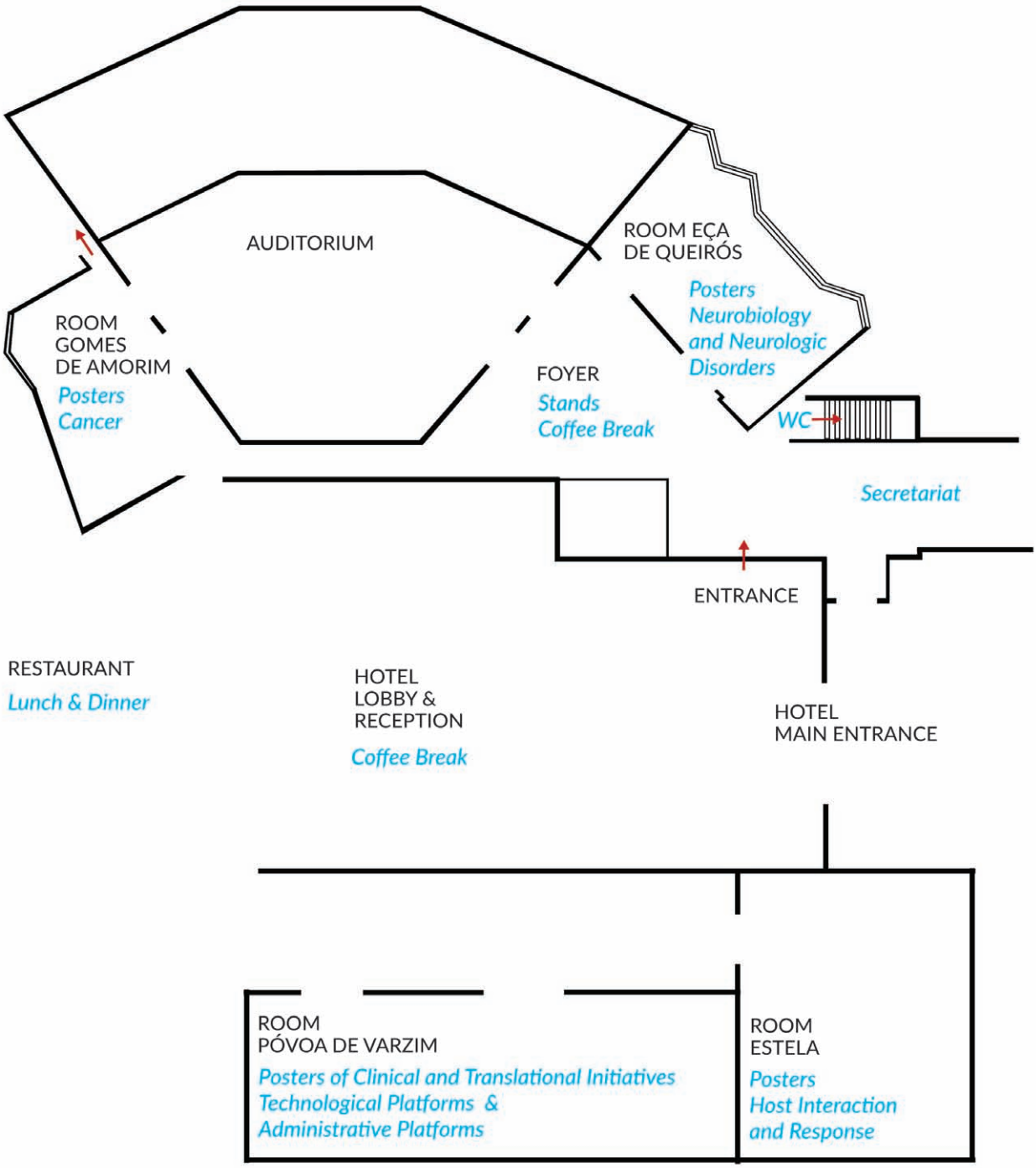
Posters of the Integrative Programme Cancer – Gomes de Amorim Room
Posters of the Integrative Programme Host Interaction and Response – Estela Room
Posters of the Integrative Programme Neurobiology and Neurologic Disorders – Eça de Queirós Room
Posters of Clinical and Translational Initiatives – Póvoa de Varzim Room
Posters of Technological Platforms – Póvoa de Varzim Room
Posters of Administrative Platforms – Póvoa de Varzim Room

Please consult your poster number in the abstract book.
Conference staff will be present to provide assistance. Authors should remain next to their poster during their poster sessions.
All posters must be placed until 14:45 on October 30 and they should be removed until 14:00 on October 31.

Internet access
Wireless Internet is available for free in the venue.

Venue

Axis Vermar Conference & Beach Hotel, Póvoa de Varzim



Thursday, 30th October

Axis Vermar Conference & Beach Hotel, Póvoa de Varzim

08:30 Registration

09:30 Welcome

10:00 **SESSION I – CANCER**
Chair: José Carlos Machado and Raquel Seruca

At the crossroads of post-transcriptional regulatory networks: the role of the RNA-binding protein MEX3A in gastrointestinal cancer
Bruno Pereira

The tumour microenvironment: the role of macrophages
Maria José Oliveira

How oncogenes and non-neoplastic components play together for colorectal cancer development
Sérgia Velho

Actin filament network remodeling during cytokinesis
Ana Carvalho

12:00 Coffee break

12:30 **PLENARY LECTURE I**
Chair: Helder Maiato
Mechanisms of mitosis and the origins of chromosomal instability in human cancer cells
Duane Compton, Geisel School of Medicine, Dartmouth, USA

13:30 Lunch

15:00 **GENERAL POSTER SESSION**
PARALLEL SESSION:
Presentation of the COHiTEC Program
Alípio da Torre, Coordinator of the Porto Edition of COHiTEC Program

16:30 Coffee break & **GENERAL POSTER SESSION**

17:00 **GENERAL POSTER SESSION**

19:00 **UPDATE ON THE I3S PROJECT**

20:00 Dinner

Friday, 31st October

09:30 SESSION II – NEUROBIOLOGY AND NEUROLOGIC DISORDERS
Chair: Deolinda Lima and Mónica Sousa

Profilin1 is required for peripheral nervous system myelination
João Relvas

Ether-phospholipids regulate Schwann cell differentiation through membrane signaling
Pedro Brites

Dopaminergic Modulation of Pain-Related Working Memory Deficit
Vasco Galhardo

nanoBiomaterials for neurosciences: one tool, many uses
Ana Paula Pêgo

11:30 Coffee break

12:00 PLENARY LECTURE II
Chair: Vitor Costa
Keeping mitochondria in shape: a matter of life, death and differentiation
Luca Scorrano, Venetian Institute of Molecular Medicine, Italy

13:00 Lunch

14:30 SESSION III – HOST INTERACTION AND RESPONSE
Chair: Anabela Cordeiro-da-Silva and Pedro Rodrigues

Inflammation in Bioengineering: from prejudice to alliance
Mário Barbosa

BioEngineered surfaces to fight infection
Cristina Martins

AIP56, an apoptogenic AB toxin targeting NF-kB
Ana do Vale

Unravelling the effect of *Listeria monocytogenes* on the control of the host cell cycle progression
Didier Cabanes

16:30 Coffee break

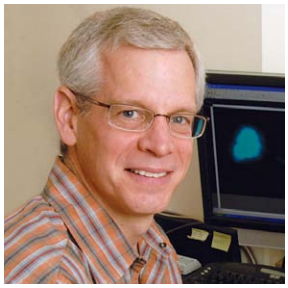
17:00 PLENARY LECTURE III
Chair: Pedro Granja

High throughput materials discovery: a new class bacterial resistant polymers and novel synthetic stem cell culture substrates
Morgan Alexander, Director of Interface and Surface Analysis Centre, Faculty of Science, School of Pharmacy, University of Nottingham, UK

18:00 FINAL REMARKS

PLENARY LECTURES

PLENARY LECTURE I



Duane Compton

Geisel School of Medicine, Dartmouth, USA

Duane Compton serves as Interim Dean of the Geisel School of Medicine at Dartmouth, where he is also the Senior Associate Dean for Research and a Professor of Biochemistry.
Dr. Compton received his PhD from the University of Texas Health Science Center at Houston in 1988 and completed his postdoctoral training in the field of cell biology at the Johns Hopkins University School of Medicine.
He was recruited to the faculty at Dartmouth in 1993, and is a leader in graduate and medical student education and a distinguished National Institutes of Health-funded researcher.

In his research program, Dr. Compton focuses on understanding how cancer cells acquire abnormal chromosome numbers and how those alterations influence cancer cell growth. He has published more than 70 articles, and images of his work have been displayed on the cover of 15 different journals. He serves on national committees with the American Society for Cell Biology and American Association of Medical Colleges and reviews grant applications for the National Institutes of Health and the American Cancer Society. He is an elected fellow of the American Association for the Advancement of Science. In 2013 he earned a MERIT award from the NIH to provide long-term support for his research program.

Dr. Compton is committed to graduate and medical education. He has graduated 14 PhD students and has served on committees for more than 50 graduate students. He received the Graduate Faculty Mentor Award in 2007. He spent six years as Biochemistry course director for the first year medical student curriculum and received the Distinguished Lecturer Award in 2004. He was a member of the inaugural class of faculty elected to the Geisel Academy of Master Faculty Educators in 2012. Dr. Compton is an avid snowboarder who lives with his wife in Lyme, NH.

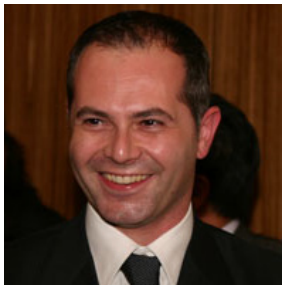
Mechanisms of mitosis and the origins of chromosomal instability in human cancer cells

Duane Compton

Geisel School of Medicine, Dartmouth, USA

Most solid tumors are aneuploid and many have defects in chromosome segregation causing them to frequently mis-segregate whole chromosomes in a phenomenon called chromosomal instability (CIN). Previously, we used live cell imaging and clonal cell analyses to evaluate chromosome segregation in chromosomally stable and unstable human cells. These experiments revealed that the most common mitotic defect in tumor cells with CIN is the persistence of erroneous attachment of spindle microtubules to kinetochores on chromosomes (i.e. merotely). In normal diploid cells erroneous attachments arise spontaneously but are efficiently corrected by high dynamic turnover of kinetochore-microtubule attachments to preserve genome stability. However, kinetochore-microtubule attachments in cancer cells with CIN are inherently more stable that those in normal diploid cells preventing efficient error correction. The observed differences in attachment stability account for the persistence of mal-attachments into anaphase, where they cause chromosome mis-segregation. Importantly, strategically decreasing the stability of kinetochore-microtubule attachments suppresses CIN and restores faithful chromosome segregation fidelity to aneuploid cancer cells. These experiments unveil a homeostatic control mechanism for regulating kinetochore-microtubule attachments during mitosis. Moreover, we have developed cell permeable small molecules that specifically suppress chromosome mis-segregation in CIN tumor cells without affecting diploid cells and we have discovered that tumor cells rapidly acquire adaptive resistance to targeted therapy.

PLENARY LECTURE II



Luca Scorrano

Venetian Institute of Molecular Medicine, Italy

Born in 1971, Luca Scorrano received his MD (1996) and PhD (2000) from the University of Padua (IT)Medical School mentored by P. Bernardi.
He then spent a HFSP funded postdoctoral fellowship at the DFCI, Harvard Medical School, Boston (USA).
He returned in Italy in 2003 to become an Assistant Scientist of the Dulbecco-Telethon Institute at the Venetian Institute of Molecular Medicine, Padua.
In 2007 he moved to Switzerland where he was appointed Full Professor at the Dept. of Cell Physiology and Metabolism, University of Geneva.
In 2013 he returned to Italy to become the Chair of Biochemistry at the Dept. of Biology, University of Padua, and from May 2014 the Scientific Director of the Venetian Institute of Molecular Medicine. The recipient of many national and international award, Luca is EMBO Member since 2012 and serves on the panel of many Journals including EMBOJ and CDD.
His laboratory combines genetics, mitochondrial physiology, advanced imaging and biochemistry to study the role of mitochondrial shape in health and disease.

Keeping mitochondria in shape: a matter of life, death and differentiation

Luca Scorrano

Venetian Institute of Molecular Medicine, Italy

In the last years, mitochondrial ultrastructural and morphological changes have been implied in the control of several physiological and pathological changes, including the progression of apoptosis. However, the role of mitochondrial dynamics in the control of complex cellular cues and in response to reversible and irreversible cellular damage is not clarified. Today we will overview the key experiments that shed light on the role of mitochondrial shape and ultrastructure in cell physiology and we will present our recent data obtained in genetic models of ablation and up-regulation of the key mitochondrial shaping proteins Optic atrophy 1 (Opa1) and mitofusin in the mouse and in embryonic stem cells. The *in vivo* experiments of tissue damage by inducing atrophy, apoptosis, ischemia/reperfusion or nuclear defects in mitochondrial respiratory chain indicate that the master cristae biogenetic regulator Opa1 can prevent multiple forms of tissue damage by controlling mitochondrial cytochrome c release and metabolic efficiency. Ablation of Mitofusin 1 and 2 in the embryonic mouse heart, or gene-trapping of Mitofusin 2 or Opa1 in mouse embryonic stem cells (ESCs), arrested mouse heart development and impaired differentiation of ESCs into cardiomyocytes. Gene expression profiling revealed roles for TGFβ/BMP, SRF, GATA4, and Mef2 differentiation factors, linked to increased Notch1 activity that, unexpectedly downstream of the Ca^{2+}-dependent phosphatase calcineurin, impaired ESC differentiation.

Orchestration of cardiomyocyte differentiation by mitochondrial morphology reveals how mitochondria, Ca^{2+} and calcineurin interact to regulate Notch1 signaling. Our data indicate that the mitochondrial shape and ultrastructure dictate organelle function and complex tissue responses ranging from death to differentiation

PLENARY LECTURE III

Morgan Alexander

Director of Interface and Surface Analysis Centre,
Faculty ofScience, School of Pharmacy,
University of Nottingham, UK

Morgan Alexander is Professor of Biomedical Surfaces at the School of Pharmacy, University of Nottingham and a Royal Society-Wolfson Research Merit Award holder. He received his Bachelor of Science in Materials in 1988 and his PhD from the same department at The University of Sheffield in 1992.

He is a member of the Laboratory of Biophysics and Surface Analysis (LBSA) which consists of 11 independent academics with complementary expertise in solving fundamental and applied biomolecular, biomaterial and (bio)formulation problems.

His work involves developing materials for application in biological environments and characterising relationships between the surface and biological response. Understanding these relationships is critical in the development of biomaterials and is the theme running through his group's work across a variety of biomedical application areas spanning bacterial adhesion to controlling stem cell response. This work is funded by major awards from the Wellcome Trust and the Engineering and Physical Sciences Research Council (EPSRC).

High throughput materials discovery: a new class bacterial resistant polymers and novel synthetic stem cell culture substrates

Morgan Alexander

Director of Interface and Surface Analysis Centre, Faculty of Science, School of Pharmacy, University of Nottingham, UK

Polymer micro arrays have proven to be useful tools for the discovery of new synthetic materials to support and guide cells.[1] This high throughput (HT) materials discovery approach is attractive because the paucity of understanding of the cell-material interface hinders the ab initio rational design of new materials.[2] The large number of polymer chemistries that can be investigated on a single polymer micro array act as a wide "net" in the search for materials that can achieve a certain cell response. Micro array hits are the starting point from which new materials may be developed.

Combinatorial acrylate libraries formed on standard glass slides were presented as a HT platform by Anderson and Langer of MIT.[3] To complement HT materials screening, we developed the approach of HT surface characterisation employing a range of analytical techniques in collaboration with the MIT group.[4] This surface characterisation step is necessary to directly relate the effect of the material on attached cells to the actual surface on which they sit, and to enable effective scale up from micro array to culture ware dimensions. Application of chemometrics, to handle the large amounts of complex data, reveals the importance of certain surface moieties, guiding the process of materials discovery and increasing our understanding of the cell-material interface.

We have applied this approach to the identification of materials which resist bacterial attachment and biofilm formation with application in the reduction of medical device centred infection, such as for urinary catheters.[5,6] In the mammalian cell field, we have identified materials which show promise as synthetic substrates for pluripotent stem cell culture.[7,8] These materials require pre-treatment with expensive proteins such as vitronectin, a constraint which limits their commercialisation.[9] In this talk, HT screening of arrays with greater chemical diversity than before, incorporating up to 140 monomers[10], is reported which leads to the identification of materials which do not require protein pre-treatment. Materials which support differentiation to cardiomyocytes with relatively mature phenotype which have potential application in *in vitro* toxicology screening will also be presented.

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[10] Celiz, A., Smith, J., Patel, A., Langer, R., Anderson, D., Barrett, D., Young, L., Davies, M., Denning, C. & Alexander, M. New Materials for Human Pluripotent Stem Cell Culture: High Throughput Discovery and Prediction of Polymers that Promote Cell Attachment. Biomaterials Science 2, 1604-1611(2014).

ORAL COMMUNICATIONS

01
At the crossroads of post-transcriptional regulatory networks: the role of the RNA-binding protein MEX3A in gastrointestinal cancer
B. Pereira, L. David, M. Billaud, R. Almeida

02
The tumour microenvironment: the role of macrophages
Maria José Oliveira

03
How oncogenes and non-neoplastic components play together for colorectal cancer development
Velho S, Wang F, Haigis K, Seruca R

04
Actin filament network remodeling during cytokinesis
Ana Xavier de Carvalho

05
Profilin1 is required for peripheral nervous system myelination
Laura Montani, Tina Buerki-Thurnherr, Joana Paes de Faria, Jorge A. Pereira, Nuno G. Dias, Rui Fernandes, Ana F. Gonçalves, Attila Braun, Yves Benninger, Ralph T. Böttcher, Mercedes Costell, Klaus-Armin Nave, Robin J.M. Franklin, Dies Meijer, Ueli Suter, João B. Relvas

06
Ether-phospholipids regulate Schwann cell differentiation through membrane signaling
P. Brites

ORAL COMMUNICATION 01

At the crossroads of post-transcriptional regulatory networks: the role of the RNA-binding protein MEX3A in gastrointestinal cancer

B. Pereira[1], L. David[1,2], M. Billaud[3], R. Almeida[1,2]

[1]IPATIMUP – Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal, [2]FMUP – Faculty of Medicine of the University of Porto, Porto, Portugal, [3]INSERM-UJF U823, Institut Albert Bonniot, Grenoble, France

As part of a highly dynamic web of messenger ribonucleoprotein complexes that act at the level of transcript processing, localization, translation, and decay, RNA-binding proteins control virtually all aspects of RNA life. Not surprisingly, defects in their expression or activity underlie the onset of diverse pathological conditions, including cancer. Aiming at unveiling regulatory mechanisms of the intestinal transcription factor CDX2, whose deregulation is associated with gastrointestinal carcinogenesis, we have established a 3D culture system incorporating a gastric carcinoma cell line and a Matrigel extracellular matrix to better mimic the *in vivo* milieu. By fusing this cell-matrix interaction model with high-throughput genome-wide screening, we have uncovered an RNA-binding protein called MEX3A as a target putatively involved in CDX2 regulation. MEX3A is a member of the evolutionarily-conserved MEX-3 family of RNA-binding proteins which have been described as mediators of post-transcriptional regulation in different biological contexts. We have demonstrated that MEX3A has a post-transcriptional repressive function over CDX2 in gastric and colorectal cellular models. Furthermore, MEX3A impairs intestinal differentiation and polarization, affects cell cycle progression and promotes increased expression of intestinal stem cell markers, namely *LGR5*, *BMI1* and MSI1.

We are currently exploring the biological functions of MEX3A by studying its expression profile in gastrointestinal cancers and identifying new targets of interaction. We are now focusing on a putative role of MEX3A on the regulation of the LKB1-AMPK-mTOR signaling axis, which converts metabolic energy-sensing information into polarity cues and is known to be altered in different types of cancer. Our data shows that MEX3A regulates the LKB1-mTOR pathway by decreasing STRAD protein expression. It is known that STRAD and the scaffolding protein MO25 assemble into a complex with the tumor suppressor LKB1. The induced LKB1 kinase activity inhibits the mTOR pathway in an AMPK-dependent manner. In agreement, we observe decreased interaction between LKB1 and STRAD in intestinal cells overexpressing MEX3A, lower activation levels of AMPK, and increased mTOR signaling assessed by the activation of two canonic mTOR targets, phosphorylated S6 ribosomal protein and phosphorylated 4EBP1. We are employing cell line-based assays with modulation of MEX3A expression and of different elements of this signaling pathway to determine how this regulation affects cell polarity and the response to induced metabolic stress. This work will shed new light into the cell biology of metabolic response and polarity and will allow us to understand the influence that MEX3A-mediated post-transcriptional control has on carcinogenesis, with important clinical implications.

ORAL COMMUNICATION 02

The tumour microenvironment: the role of macrophages

Maria José Oliveira

INEB - Instituto de Engenharia Biomédica

Macrophages constitute one of the most abundant elements present at the tumour microenvironment. Once recruited to the tissues they differentiate into distinct inflammatory populations: a pro-inflammatory M1-like, which may exert tumour cytotoxicity, and an anti-inflammatory M2-like which may promote tissue repair and remodelling, angiogenesis and tumour progression. Tumour-associated macrophages, expressing several anti-inflammatory functions, are considered allies of tumour progression in several types of cancer, although their role on gastric and colorectal carcinomas is still poorly understood.

We have recently described that human naïve macrophages stimulate gastric and colorectal cancer cell invasion, motility/migration and proteolysis, elucidating the underlying molecular mechanisms. siRNA and immunoprecipitation experiments revealed that these effects depend on matrix metalloproteinase (MMP) activity and on membrane recruitment and activation of epidermal growth factor receptor (EGFR) (at the residue Y^{1086}), PLC-γ and Gab1. Epidermal growth factor (EGF)-immunodepletion impaired macrophage-mediated cancer cell invasion and motility, suggesting that EGF is the pro-invasive and pro-motile factor produced by macrophages. Macrophages also induce cancer cell phosphorylation of Akt, c-Src and ERK1/2, and led to an increase of RhoA and Cdc42 activity.

Remarkably, we demonstrated that M2-like macrophages are the most efficient in promoting *in vitro* cancer cell invasion and migration. In addition, soluble factors produced by these macrophages enhanced *in vivo* cancer cell-induced angiogenesis, as opposed to their LPS-stimulated counterparts. Differences in the ability of these populations to stimulate invasion or angiogenesis cannot be explained by the EGFR-mediated signaling, since both LPS- and IL-10-stimulated macrophages similarly induce the phosphorylation of cancer cell EGFR, c-Src, Akt, ERK1/2, and p38. Interestingly, both populations exert distinct proteolytic activities, being the M2-like macrophages the most efficient in inducing matrix metalloprotease (MMP)-2 and MMP-9 activities. In addition, we are currently investigating the role of tumour-derived extracellular matrix components on macrophage differentiation and assessing the macrophage profile distribution along distinct regions of human colorectal cancer tumour resections.

Finally, we constructed Ch/γ-PGA multilayered films as IFN-γ delivery systems for the modulation of macrophage phenotype towards an anti-inflammatory and anti-tumour phenotype, providing the basis for the design of a therapeutic strategy to target macrophages at the tumour site.

ORAL COMMUNICATION 03

How oncogenes and non-neoplastic components play together for colorectal cancer development

Velho S[1], Wang F[2], Haigis K[2], Seruca R[1,3]

[1]Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal; [2]Molecular Pathology Unit, Center for Cancer Research and Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Charlestown, USA; [3]Faculdade de Medicina da Universidade do Porto, Portugal;

In cancer, mutations in the different RAS oncogenes (*H-RAS*, *N-RAS*, *K-RAS*) are frequently found, however, they preferentially associate with distinct tumor types. In colorectal cancer, *K-RAS* oncogenic mutations are extremely common (40%), whereas activating mutations in *N-RAS* occur in a small subset of the tumors (2-5%). Given the highly conserved enzymatic function of the RAS isoforms, it remains unclear what accounts for their differing mutational frequencies. Still, mutations in these two oncogenes might have redundant effects, as they are never found in the same tumor. However, it was previously shown that in the mouse colonic epithelium *K-RAS*^{G12D} induces hyper-proliferation that manifests as chronic intestinal hyperplasia and, in the context of mutant APC, strongly enhances the transition from a benign adenoma to a malignant adenocarcinoma. In contrast, *N-RAS*^{G12D} does not affect basal homeostasis or tumor progression, but instead inhibits the ability of intestinal epithelial cells to undergo apoptosis in response to stress. These observations favor the idea that *K-RAS* and *N-RAS* mutations are mutually exclusive, not because they are redundant, but because they are selected for under distinct tumorigenic contexts.

Our aim was to unravel if, and in which context the anti-apoptotic effect of activated *N-RAS* contributes to the initiation and progression of colorectal cancer.

Using genetically engineered mouse models of colonic carcinogenesis, we found that mutant *N-RAS* promotes tumorigenesis in the context of inflammation. We verified that the pro-tumorigenic nature of mutant *N-RAS* is related to its anti-apoptotic function, which is mediated by activation of a non-canonical mitogen-activated protein kinase pathway that signals through STAT3. As a result, inhibition of mitogen-activated protein kinase cascade selectively induces apoptosis in autochthonous colonic tumors expressing mutant *N-RAS*. The translational significance of this finding is highlighted by our observation that *N-RAS* mutation correlates with a less favorable clinical outcome for patients with colorectal cancer. In summary, our study links the anti-apoptotic function of mutant *N-RAS* to its ability to promote colorectal cancer in an inflammatory context, and pinpoints a therapeutic strategy for *N-RAS* mutant colorectal cancers. In addition, it also points out the influence of the microenvironment in dictating the role of oncogenes in tumor development.

ORAL COMMUNICATION 04

Actin filament network remodeling during cytokinesis

Ana Xavier de Carvalho

IBMC - Instituto de Biologia Molecular e Celular

The dynamic remodeling of the actin cytoskeleton is essential for a variety of cellular functions, including polarity establishment, cell-cell adhesion, migration, invasion and division. This is because the organization of actin filaments in different configurations is key to define cell shape and behavior. The dynamics of the actin filament network depend on the action of actin regulators such as actin filament nucleators. The two major nucleators are the Arp2/3 complex and the formins. Branched or non-branched actin filament networks form as a result of ARP2/3 or formin complex-dependent actin filament nucleation, respectively. We are interested in understanding how these two types of actin network contribute to cytokinesis, the process that completes cell division by partitioning the contents of the mother cell into two daughter cells. Our results show that both actin nucleators are important for cytokinesis. Cytokinesis requires the assembly and subsequent constriction of a contractile ring and we found that branched and non-branched networks act consecutively during contractile ring assembly. Our current goal is to understand how the activity of the two nucleators is coordinated in space and time and whether the two nucleators interfere or collaborate with one another.

ORAL COMMUNICATION 05

Profilin1 is required for peripheral nervous system myelination

Laura Montani[1,2], Tina Buerki-Thurnherr[2,3], Joana Paes de Faria[1], Jorge A. Pereira[2], Nuno G. Dias[1], Rui Fernandes[1], Ana F. Gonçalves[1,2], Attila Braun[4], Yves Benninger[2], Ralph T. Böttcher[4], Mercedes Costell[4,5], Klaus-Armin Nave[6], Robin J.M. Franklin[7], Dies Meijer[8], Ueli Suter[2], João B. Relvas[1,2,9]

[1]Instituto de Biologia Molecular e Celular, Porto, Portugal [2]Institute of Molecular Health Sciences, Department of Biology, Swiss Federal Institute of Technology, ETH Zurich, Switzerland [3]EMPA, Swiss Federal Laboratories for Materials testing and Research, Materials-Biology Interaction, St. Gallen, Switzerland [4]Max-Planck Institut für Biochemie, Martinsried, Germany [5]Department of Biochemistry and Molecular Biology, Universitat de València, Spain [6]Max-Planck für Experimentelle Medizin, Göttingen, Germany [7]Wellcome Trust-Medical Research Council, Stem Cell Institute, Cambridge, UK [8]Erasmus MC, Rotterdam, Netherlands

Myelination allows rapid saltatory propagation of action potentials along the axon and is an essential prerequisite for the normal functioning of the nervous system. During peripheral nervous system (PNS) development, myelin-forming Schwann cells (SC) generate radial lamellipodia to sort and ensheath axons. This process requires controlled cytoskeletal remodeling, and we show that SC lamellipodia formation depends on the function of profilin1 (Pfn1), an actin-binding protein involved in microfilament polymerization. Profilin is inhibited upon phosphorylation by ROCK, a downstream effector of the integrin-linked-kinase pathway. Thus, a dramatic reduction of radial lamellipodia formation is observed in SCs lacking ILK or treated with the Rho/ROCK activator lysophosphatidic acid. Knocking-down Pfn1 expression by lentiviral-mediated shRNA delivery impairs SC lamellipodia formation *in vitro*, suggesting a direct role for this protein in PNS myelination. Indeed, SC-specific gene ablation of profilin1 in mice led to profound radial sorting and myelination defects, confirming a central role for this protein in PNS development. Our data identify profilin1 as a key effector of the integrin-linked-kinase/rho/ROCK pathway. This pathway acting in parallel with integrinβ1/LCK/Rac1 and their effectors critically regulates SC lamellipodia formation, radial sorting and myelination during peripheral nervous system maturation.

ORAL COMMUNICATION 06

Ether-phospholipids regulate Schwann cell differentiation through membrane signaling

P. Brites
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Rhizomelic Chondrodysplasia Punctata (RCDP) is a complex developmental disorder caused by a deficiency in the biosynthesis of ether-phospholipids. Plasmalogens, the most abundant form of ether-phospholipids, are a unique class of membrane glycerophospholipids containing a fatty alcohol with a vinyl-ether bond at the sn-1 position of the glycerol backbone. Although high levels of these phospholipids are found in nervous tissue, and their deficiency is associated with several neurodegenerative disorders, how and to what extent a plasmalogen deficiency causes myelin defects is unknown.

Using mouse models of RCDP, we unraveled that a deficiency in plasmalogens causes defects in Schwann cell development that impair their ability to interact with axons, initiate myelination, and assemble normal myelin. Mutant mice also develop progressive ataxia and hindlimb paralysis, due to demyelination and axonal degeneration. Mechanistically, we identified a defect in protein kinase B (AKT) phosphorylation and in its signaling pathway, causing overt activation of glycogen synthase kinase 3beta (GSK3beta). Treatment with GSK3beta inhibitors is able to restore the defects in Schwann cell differentiation bypassing the defects in

Our results demonstrate the requirement of plasmalogens for the correct and timely differentiation of Schwann cells and for the process of myelination. In addition, our work indentifies a mechanism by which a membrane phospholipid mediates neuropathology, implicating plasmalogens as regulators of membrane and cell signaling.

ORAL COMMUNICATION 07

Dopaminergic Modulation of Pain-Related Working Memory Deficit

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Chronic pain is known to trigger profound alterations in forebrain circuitry and induce cognitive deficits that range from memory and attentional impairments, to mood and sleep disorders. There is a current large interest in understanding the interplay occurring between the fucntional changes in forebrain circuitry and the onset of cognitive impairment during chronic pain conditions. Our lab is working to uncover the neurobiological mechanisms that are altered by chronic pain, paying special interest to the forebrain areas that constitute the reward-aversion circuitry and in knowing to what extent the modulation of the dopaminergic transmission mat have therapeutic potential in the reversal of pain-induced disorders.

In this presentation at the annual retreat, we will summarize the studies that we have conducted in the last five years, presenting results from two lines of research: one addressing problems in the stability of hippocampus-based spatial memories, and another addressing problems arising in the processes of prefrontal-based emotional decision-making.

The hippocampus is a brain area that plays a crucial role in the interplay between memory processing and stress modulation, and that is known to be critically important to the acquisition and extinction of behaviours related to highly aversive stimuli. We have shown that the onset of chronic pain disrupts the stability of receptive fields in hippocampus place cells [1] and that it disrupts the functional connectivity frequency coherency within the frontal-thalamus-hippocampus pathway that is relevant for spatial working memory [2,3]. Recently we described how the loss this functional connectivity within the hippocampus may be restored by modulation of dopaminergic neurotransmission [4].

In 2007 we developed of a novel decision-making task for rodents that is able to mimic the complex behavioral traits observed in humans faced with uncertain alternatives, such as gambling tasks. Using this novel test, we have shown that control animals prefer the choice that signals lower but more probable rewards, while animals with lesions in the orbitofrontal cortex perform this task in a very distinct way, favoring risky decisions that sometimes result in large jackpot-like rewards. More recently, we have extended our results in a series of papers in which we showed that pain induces performance deficits akin to orbitofrontal lesions [5], that these pain-induced changes may be reversed by modulation of the fronto-amygdalar pathway [6], and that orbitofrontal neural activity during performance in the rodent gambling task is distinct in control versus neuropathic pain animals [7]. In recent still unpublished studies we show that systemic and intra-PFC modulation of dopamine receptors reverses the risk-taking impulsiveness caused by the onset of pain. Our results suggest that performance impairment is caused by the lack of balance in dopamine levels: administration of either agonists or antagonists in control animals decreases performance, while in pain animals the antagonist restores performance and the agonist decreases it further.

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ORAL COMMUNICATION 08

nanoBiomaterials for Neurosciences: one tool, many uses

Ana Paula Pêgo

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The impact of disease or a physical injury on the nervous system can have devastating consequences to the individual and most frequently significant personal, societal and economical repercussions. Nervous system problems are common and encompass a large spectrum of traumatic injuries, diseases, tumours or iatrogenic lesions (e.g. after radio- or chemotherapy). Among these, neurodegenerative diseases like Alzheimer and Parkinson are becoming significantly prevalent in our aging population.

The poor regenerative capacity, particularly in the case of the central nervous system (CNS), cannot be attributed to an intrinsic inability of neurons to sprout and re-grow after injury, as these axons are able to regenerate in the presence of a permissive growth environment. One of the challenges facing neuroscientists is the development of effective therapies that can spur the regenerative capacity of the nervous system based on the advances achieved in basic research.

The application of (nano)biomaterials to neuroscience has given so far a substantial contribute to the development of implantable and interfaceable devices dedicated to the restoration of a number of neural functions. But the contribute of biomaterials in the context of nervous system regenerative medicine is still in its infancy with expected impact both in the framework of tissue engineering, drug delivery and imaging approaches. We have been dedicated to using nano-enabled solutions to the design of new therapeutic approaches towards the enhancement of the process of nerve regeneration.

Two main strategies are being followed and will be discussed:

- the development of materials designed at the nanoscale (hydrogels and synthetic elastomers) for the preparation of hybrid grafts to be used in nerve defect correction[1,2];
- the design of biomaterial-based nanoparticles for targeted nucleic acid delivery to neurons[3].

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ORAL COMMUNICATION 09

Inflammation in Bioengineering: from prejudice to alliance

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Several generations of biomaterials have been used over the last 50 years. Initially, biomaterials should be as inert as possible, in the sense that they should not suffer degradation and lead to accumulation of toxic elements in the surrounding tissues. This was the case of metals and hard ceramics. Biomaterials degradation led often to implant failure and subsequent removal of the device.

The inflammatory response that occurs upon implantation of any device is responsible for the formation of a collagen-rich fibrous capsule around the device, which becomes isolated from the rest of the tissues. In case of permanent implants this capsule may contribute to a decrease in the adverse effects associated with the biomaterials degradation, but it may also lead to deficient transmission of mechanical loads (e.g. orthopaedic and dental implants). Also, whenever a sensing device (e.g. for measurement of glucose) or an electrode (e.g. for neurostimulation) is surrounded by a fibrous capsule a significant decrease in input and output signals is observed.

These two situations led to the concept that inflammation is "bad" in bioengineering applications of biomaterials.

Therefore, minimizing the inflammatory reaction associated with biomaterials (called a foreign body reaction) has been the objective of numerous studies. In the limit, a biomaterial should not be recognized by the immune system, thus becoming "invisible". This concept of stealthy biomaterials (that travel in the body like invisible airplanes) has emerged and intensive research has been directed into this area, with applications in drug delivery by nano- and micro-particles and biosensors.

Whenever a degradable biomaterial is used to assist tissue regeneration, inflammation is also present, but minimizing it might result in poor tissue remodeling. Although the concept of an "optimal" degree of inflammation has been advocated, this is still vaguely translated into biomaterials with immunomodulatory properties. The concept of a biomaterial designed to act primarily on the behavior of immune cells and not of other cells (e.g. stem cell) is new and goes against the former paradigm of minimizing inflammation.

In this talk examples of research carried out at our group, that illustrate the two approaches, will be presented.

ORAL COMMUNICATION 10

BioEngineered Surfaces to Fight Infection

M Cristina L Martins

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Helicobacter pylori infection is considered to be a primary risk factor for the development of gastric ulcer and gastric cancer (1). This bacterium is one of the most common infectious agents in the world, colonizing the gastric mucosa of over 50% of the World's population (2). Increase in antimicrobial resistance to standard antibiotic treatments (3) demonstrated the need of alternative therapies.

Our team has been working on an innovative strategy for *H. pylori* infection treatment, based on the clearance of this bacterium from the stomach using specific *H. pylori*-binding biomaterials (4).

The creation of specific biomaterials needs a precise control of the interface between the biomaterial surface and the bacterium. Self-assembled monolayers (SAMs) are a useful tool for fundamental studies regarding these interactions, since their composition can be controlled at nanoscale and due to their simple method of production.

Adhesion of *H. pylori* to the gastric epithelium is mediated by bacterial adhesins that recognize specific glycan structures expressed in the gastric mucosa. The blood group antigen binding adhesin (BabA) recognizes difucosylated antigens such as Lewis B (Leb) (5), while the sialic acid binding adhesin (SabA) recognizes sialylated glycoproteins, such as sialyl-Lewis x (sLex) (6).

SAMs with capacity to bind *H. pylori* via specific adhesin-glycan recognition were successfully engineered (7). Glycans selected using SAMs were thereafter immobilized onto mucoadhesive biomaterials demonstrating their capacity to selectively attract and bind *H. pylori* through its specific adhesins (8).

During this presentation, this work will be used to exemplify the potentialities of nanotechnology in the development of innovative strategies to fight infection

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ORAL COMMUNICATION 11

AIP56, a bacterial AB toxin targeting NF-kB

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Bacterial AB toxins are outstanding autonomous molecular devices that use highly sophisticated mechanisms to reach and enzymatically modify key cytosolic components of host cells to favor microbial infection. AIP56 (apoptosis-inducing protein of 56 kDa) is the major virulence factor of *Photobacterium damselae piscicida* (*Phdp*), a Gram-negative bacterium that causes septicemic infections in economically important marine fish species. During infection, AIP56 triggers apoptosis of host macrophages and neutrophils through a process that culminates with secondary necrosis of the apoptotic cells, contributing to the necrotic lesions observed in the diseased animals. Research performed at our laboratory revealed that AIP56 is an AB toxin with zinc-metalloprotease activity towards NF-κB p65. Similarly to other AB toxins, AIP56 is composed of a catalytic A subunit that holds the enzymatic activity bound to a B subunit that binds to cell receptor(s) and assists delivery of the toxin into the target's cell cytosol. Recently, we found that mouse bone marrow derived macrophages are susceptible to AIP56 intoxication and defined the mechanism involved in the entry and intracellular traffic of the toxin, showing that it is conserved between fish and mammalian cells. The observation that mammalian cells are susceptible to AIP56 confers biotechnological potential to the toxin, because aberrant activation of NF-kB is associated to many inflammatory diseases and cancers and thus, NF-κB constitutes a good target for anti-inflammatory and anti-cancer drugs.

ORAL COMMUNICATION 12

Unraveling the effect of *Listeria monocytogenes* on the host cell cycle progression

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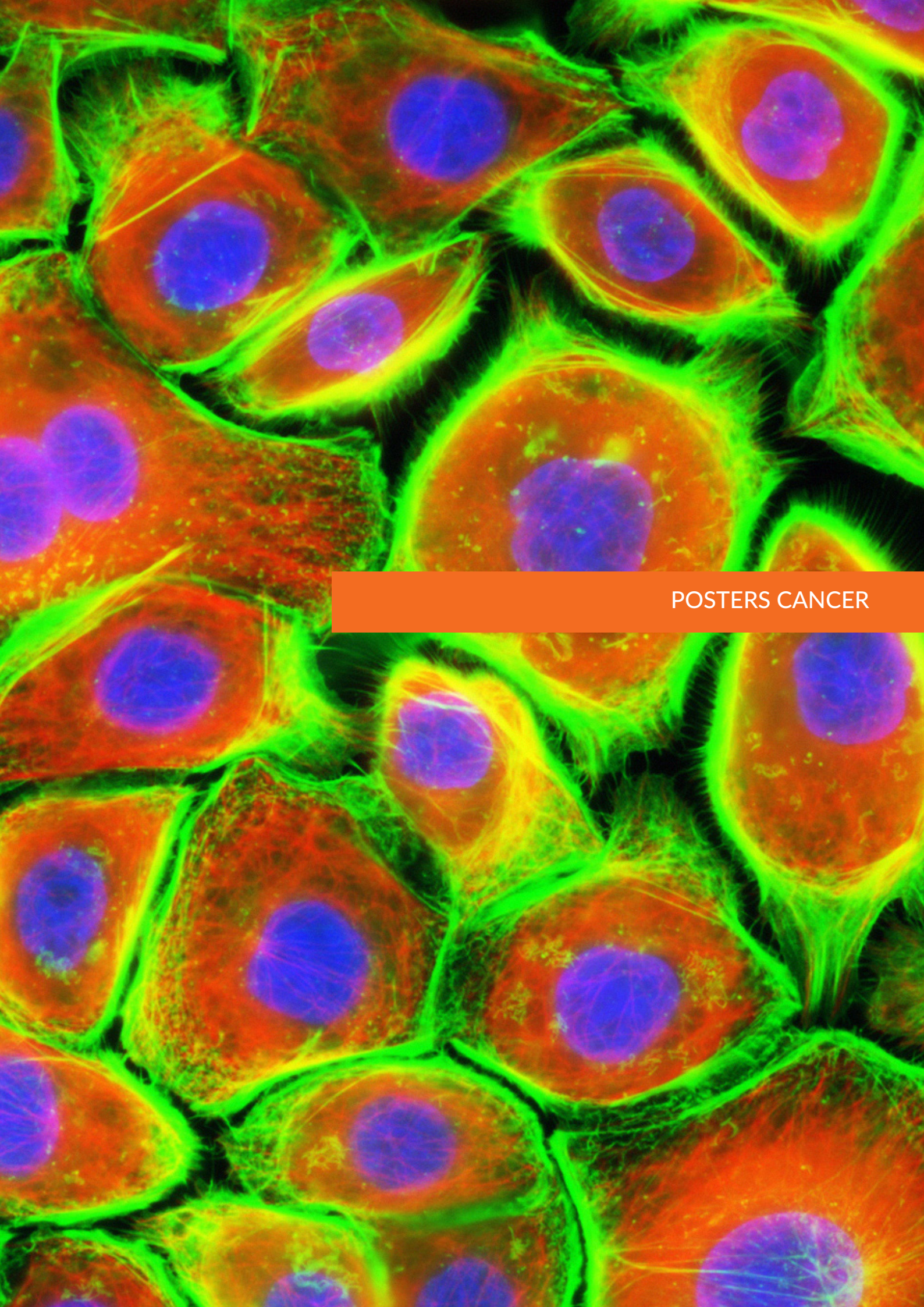
Listeria monocytogenes is a facultative intracellular foodborne pathogen able to cross tight human barriers and spread from cell to cell, causing listeriosis, a severe human disease. *Listeria* employs an arsenal of virulence factors to invade, survive, and multiply in both phagocytic and non-phagocytic cells, hijacking host-signaling pathways to establish and sustain infection. Although *Listeria* remains mostly cytosolic, its effects extend to the nucleus by interfering with histone modifications and chromatin-regulatory factors to modulate host gene expression. However, the interplay between *Listeria* and the host cell cycle was understudied. We thus investigated if *Listeria* could interfere with the host cell cycle to create a suitable replication niche. We will show here how *L. monocytogenes* induces host DNA damage and delays the host cell cycle to promote infection and discuss potential long-term effects regarding cancer and host development.



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CANCER | POSTER 01

Cancer Prevention Education: Building the Future Through School Community

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Cancer is a global health challenge, being one of the worldwide leading causes of death. Recent data clearly shows that cancer burden is steadily increasing, reflecting the populations ageing, the reduced cancer awareness and the lack of impactful cancer prevention strategies [1-3]. The majority of cancer education programs are promoted by healthcare professionals, such strategy limits the scale and scope of the interventions. Schools have a great potential to play a leading role in health prevention programs. The school environment can promote cancer prevention initiatives that affect not only teachers and adolescents but also the whole community [4]. We developed an innovative education model for cancer prevention based upon high school biology teachers. A training program targeted to these educators will provide them the necessary competences to the development and implementation of cancer prevention projects in schools. This training encompasses different educational sessions ranging from cancer biology to prevention campaigns design. To validate this novel approach, we assessed the evolution of teachers and students cancer knowledge and perceptions using a questionnaire applied in three different moments of the training: before (pre-test), immediately after (post-test), and one year after (follow-up) [5]. Preliminary results show a significant increase on cancer literacy (knowledge and perceptions) of trained teachers. Furthermore, teachers were also proficient to develop and deliver impactful cancer prevention campaigns among their students. The results gather so far prove the relevance of this new cancer prevention strategy. This model might be a significant contribution to a new paradigm of health education based on focused interventions, mediated by non-healthcare professionals in restricted targets (e.g. school community).

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CANCER | POSTER 02

Impairment of glucose uptake in human first trimester trophoblasts by xanthohumol, mediated by tyrosine kinases, mammalian target of rapamycin and C-JUN N-terminal kinases intracellular pathways, has consequences upon the process of placentation

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The aim of this work was to investigate whether modulation of glucose transport by some polyphenols in first trimester extravillous trophoblasts (EVTs), which are cells that regulate the process of placentation, affects cell viability, proliferation, growth and migration capacity.

To do this, glucose uptake by HTR-8/SVneo human first-trimester EVT cell line and its modulation by selected polyphenols was studied by measurement of cellular incorporation of ^{3}H-2-deoxy-D-glucose (^{3}H-DG).

Additionally, the effect of XH upon glucose cellular metabolism (extracellular lactate quantification) and upon the viability (lactate dehydrogenase leakage assay), proliferation (^{3}H-thymidine cellular incorporation), culture growth (sulforhodamine B (SRB) assay) and migration capacity (wound-healing assay) of HTR-8/SVneo cells were studied. Finally, MCT-mediated transport was measured by quantification of ^{14}C-butyrate (^{14}C-BT) uptake by HTR-8/SVneo cells.

Acutely (30 min), the polyphenols quercetin, epigallocatechin-3-gallate, xanthohumol (XH) and resveratrol concentration-dependently inhibited ^{3}H-DG uptake by inhibiting both GLUT and non-GLUT-mediated uptake. XH was found to be the most potent inhibitor of ^{3}H-DG uptake by HTR-8/SVneo cells and, at 100 µM, it significantly reduced the *Vmax* while not affecting the K*m* of ^{3}H-DG uptake.

Chronically (24h), XH (5 µM) also inhibited ^{3}H-DG uptake again significantly reducing the *Vmax* while not affecting the K*m*. This inhibitory effect of XH was reversed by rapamycin, genistein and SP 600125, indicating that the XH inhibitory effect upon ^{3}H-DG uptake is mediated by the mammalian target of rapamycin (mTOR), tirosine kynases (TK) and c-Jun N-terminal kinases (JNK) intracellular pathways, respectively.

In addition, XH (24h; 5 µM) decreased HTR-8/SVneo cell viability, proliferation, culture growth and migration capacity. Importantly, the effects of XH upon cell viability and culture growth were mimicked by low extracellular glucose concentration and reversed by high extracellular glucose concentration.

Finally, acute (30 min; 100 µM) and chronic XH (24 h; 5 µM) induced an increase in extracellular lactate levels over time, suggesting an inhibition of MCT-mediated transport which was confirmed by the observation that XH inhibits ^{14}C-BT (10 µM) uptake.

Altogether, these results show that XH potently and non-competitively inhibits glucose uptake by EVTs, an effect that seems to be mediated by mTOR, TK and JNK intracellular pathways. Our results also show that the cytotoxic and antiproliferative effects of XH over EVTs are mainly due to glucose deprivation.

We thus suggest that the polyphenol XH may interfere with glucose delivery to the fetus which may have consequences over the process of placentation.

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Modulation of angiogenic pathways by 8-prenylnaringenin involvement of estrogen receptors

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Introduction: 8-Prenylnaringenin (8PN) is a polyphenol and a powerful phytoestrogen with a binding affinity to estrogen receptors (ER) alpha three times higher than ERβ [1]. 17β-Estradiol stimulates angiogenesis [2]. Recent work has attributed angiogenic properties to 8PN [3], but its effect on the modulation of angiogenic process is not consistent.

Objectives: To evaluate the effect of 8PN on angiogenic pathways and to verify the dependence on the activation of the ER.

Methodology: Human umbilical vein endothelial cells (HUVEC) were stimulated with vascular endothelial growth factor (VEGF) and treated with 10μM 8PN. ERα and ERβ gene expression was evaluated by RT-PCR. HUVEC were also treated with an ERβ antagonist (PHTPP). VEGFR2, pAkt, pERK 1/2 and Tie2 expression was quantified by western blotting. Ang2 was evaluated by ELISA assay. The modulation of angiogenic pathways by 8PN was confirmed *in vitro* by quantification of tubular structures formed by HUVEC cultivated in matrigel. Quantifications are expressed as mean ± SEM. Results were evaluated by ANOVA followed by Bonferroni. A difference between experimental groups was considered significant whenever p≤ 0.05.

Results: HUVEC only transcribed ERβ. 8PN tends to increased the expression of VEGFR2 (128.8 ± 16.1%) in a process apparently dependent on ERβ and improved the expression of pERK (127.6 ± 16.8%) in a process dependent on ERβ. It stimulated the release of Ang2 (122.3 ± 0.3%) and the expression of Tie2 (140.6 ± 6.1%) independently of ERβ and didn't affect the expression of pAkt (98.8 ± 6.7%). Matrigel assay showed an increase in the formation of tubular structures (143.2 ± 4.4%) after treatment with 8PN.

Conclusion: 8PN manifested pro-angiogenic properties involving pathways regulated by binding to ERβ and pathways independent of this stimulation. This finding can be interesting, considering pathologies associated to poor angiogenesis, as myocardial ischemia, peripheral arterial disease and neurological diseases.

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CANCER | POSTER 04

Anti-Mullerian Hormone and Oocyte Secreted Factors of Human Follicular Fluid in the assessment of infertility disorders

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Infertility, defined as the absence of conception after a year of regular and unprotected intercourse is currently seen as a public health problem that motivates an increased demand for medical assistance.

The assessment of oocyte quality is of major concern for the success of medically assisted procreation techniques. Indeed, in an attempt to indirectly evaluate oocyte quality, attention has been focused in the composition of follicular fluid (FF). FF presents chief importance in the follicular microenvironment, considering that it contains proteins involved in the modulation of oocyte maturation and ovulation. Moreover, FF is easily available during oocyte pick-up in medical assisted reproduction techniques, constituting an optimal source on non-invasive biochemical predictors of oocyte quality and further success of infertility treatment.

Among the molecules involved in the intrafollicular communication, are the oocyte secreted factors (OFF's), including the growth differentiation factor 9 (GDF-9) and the bone morphogenetic protein 15 (BMP-15). Both factors belong to the transforming growth factor β family (TGF-β) but, whilst GDF-9 plays a key role in the follicular growth, BMP-15 stimulates granulosa cells proliferation. Another molecule from TGF-β family normally detectable in FF is Anti-mullerian Hormone (AMH). It is expressed by the cells from the granulose layer of primary and secondary follicles, exerting an inhibitory action in the beginning of primary follicle growth.

In the present study, we intended to evaluate the expression of GDF-9, BMP-15 and AMH in ovarian follicules of women that present disorders that may compromise the success of infertility treatment. To achieve this goal we compared levels of these factors in FF samples obtained from women with endometriosis, women with unexplained infertility and controls (male factor infertility).

FF samples obtained from women aged 31-41 years (n=35) submited to medical assisted reproduction techniques, were assigned by CETI (Centro de Estudos e Tratamento de Infertilidade) – Porto. Semi-quantification of GDF-9, BMP-15 and AMH was carried out by Western Blot technique, employing specific antibodies.

A decrease in AMH levels in both pathological groups (unexplained infertility and endometriosis) when compared with controls was found, although only the variation found for endometriosis group reached statistically significance. Conversely, the levels of BMP-15 increased in both pathological groups comparatively with controls, but only significantly in the endometriosis group. No differences among groups were seen for GDF-9 levels.

From our results we conclude that endometriosis seems to relate with a decrease of AMH levels and an increase of BMP-15 levels in FF. Further investigation is however needed to validate this data, including an increased of the number of cases studied. These molecules may become markers for the improvement assisted reproductive technology success.

CANCER | POSTER 05

The molecular link between BRAF mutation and sodium iodide symporter (NIS) underexpression

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Introduction: Thyroid carcinoma (TC) is the most common endocrine malignancy. Most patients with differentiated TC have long term survival after initial surgery and radioiodine ablation therapy[1]. Radioactive iodine is effective because of the unique ability of thyroid cells to uptake iodine from plasma, a function on a mechanism mediated by sodium iodide symporter[1,2] (NIS). NIS is a glycoprotein present in the membrane of thyroid follicular cells that is responsible for the iodide uptake[1,2]. Some TC lose NIS expression and, consequently, the ability to incorporate iodide, becoming resistant to therapy. So far, no treatment has been able to restore NIS re-expression and complete functionality[2,3]. $BRAF^{V600E}$ is the most frequent mutation in papillary thyroid carcinoma (PTC)[4]. This oncogene impairs both NIS expression and its targeting to the membrane but the underlying molecular mechanisms remain unknown[4]. Few studies have evaluated the ability of MAPK inhibitors to induce NIS restoration in thyroid tumor cell lines and xenographs. The results reported to date are controversial: NIS expression and iodide uptake[5] were restored in some studies, while in others they were not[3].

This uneven results indicate that $BRAF^{V600E}$ may promote NIS impairment by a pathway other than MAPK.

Objectives: To explore the molecular mechanisms underlying $BRAF^{V500E}$ induced NIS under-expression.

Materials and Methods: A human thyroid carcinoma cell line K1 (derived from a primary PTC), harboring $BRAF^{V600E}$ mutation and with no NIS expression was used. We performed BRAF silencing by siRNA, and evaluated, by western-blot or qRT-PCR, the levels of BRAF, pERK1/2 and NIS.

Results: BRAF was efficiently silenced by siRNA at all time points (24, 36, 48 and 72 hours). NIS mRNA and protein levels were significantly higher in cells with BRAF silencing. Although BRAF remained silenced at all time points, pERK1/2 did not follow the same pattern and at 48 hours was increased in silenced cells in comparison to the scramble control.

Conclusions: NIS expression seems to be related with BRAF status but not necessarily with MAPK down regulation. Future studies are needed to understand which mechanisms are involved, in order to develop a pharmacological strategy to improve NIS expression and better patients' outcome.

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CANCER | POSTER 06

Galectin-3 is part of the cell response to stressful conditions in canine mammary tumors

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The tumor microenvironment encompasses several stressful conditions for cells such as hypoxia, oxidative stress and pH alterations. Galectin-3, a well-studied member of the beta-galactoside-binding animal family of lectins, has been implicated in tumor progression and metastasis by promoting cell-cell and cell-extracellular matrix adhesion, angiogenesis, cell proliferation and preventing tumor cell apoptosis. Its abnormal up- and down-regulated expression has been observed in several types of cancer. However, the mechanisms that regulate galectin-3 expression in neoplastic settings are not clear. In order to demonstrate the putative role of hypoxia in regulating the galectin-3 expression by canine mammary tumors (CMT), *in vitro* and *in vivo* studies were performed to evaluate its expression under hypoxic conditions. In malignant canine mammary cells, hypoxia was observed to induce an increased expression of galectin-3, a characteristic that is almost completely prevented when cells are treated with catalase. The protein increased expression was confirmed at the mRNA level. Under hypoxic conditions the expression of galectin-3 shifts from a predominant nuclear location to cytoplasmic and membrane expressions. In *in vivo* studies, galectin-3 was overexpressed in hypoxic areas of primary tumor and well-established metastases. Thus tumor hypoxia up-regulates the expression of galectin-3 that may increase tumor aggressiveness.

CANCER | POSTER 07

MCL1 alternative polyadenylation-derived isoforms regulation by microRNAs in human T cells

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Alternative polyadenylation (APA) is an important regulator of gene expression and occurs in about 70 % of human genes.[1] APA is the process by which two or more mRNAs are produced through the usage of different polyA sites that can be present both at the coding region and at the 3'UTR. Transcripts produced by the usage of distal polyA sites, and thus with longer 3'UTRs, have more microRNA (miRNAs) and RNA-binding proteins (RBPs) binding sites and are more prone to regulation.[2] A variety of studies have been done in APA field and it is now know that APA is in the origin of a variety of diseases as well as non-pathological conditions.[3] MCL1 is a gene that encodes for an anti-apoptotic protein and is essential for the development and maintenance of both B and T lymphocytes.[4] Our aim is to characterize the APA pattern of MCL1 in human T cells, and to understand the role of miRNAs in the regulation of MCL1 APA-derived isoforms. By 3'RACE mapping we show that MCL1 produces four mRNA isoforms produced by usage of four polyA signals localized in the 3'UTR. Using luciferase assays we observe that the longest mRNA originates less protein. By in silico analysis we identified several putative miRNA target sites in the MCL1 3'UTR that are highly conserved in mammals. We focused our studies in miR-92a, which targets all mRNA isoforms, miR-29b, which targets the two longest isoforms, and miR-320a and miR-17 that target the longest isoform only. By RT-qPCR, we show that these miRNAs are expressed in T cells and by western blot we demonstrated that overexpression of miRNA-92a and miR-29b downregulates MCL1 protein levels. In addition, we show that mutation of mir-17 target site in MCL1 3'UTR increases luciferase levels in reporter assays. Since MCL1 plays a major role in suppressing apoptosis, characterization of the MCL1 mRNA isoforms produced by APA and the miRNAs that regulate its expression may be a useful therapeutic tool in different clinical contexts in the future.

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CANCER | POSTER 08

Age-related reproductive outcome. Effect of specific antioxidant therapy

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Introduction: A biological process that appears to be involved in the loss of reproductive capacity is the cell redox status, namely an age-related imbalance in redox homeostasis consequent to enhanced local production or scavenging of reactive oxygen species. Thus, it is plausible that the reduced reproductive capacity observed in aged females is founded on a redox imbalance.

Aim: To test whether specific antioxidant therapy can ameliorate age-related impaired reproductive capacity.

Methods: Uterine samples of mice aged 11–15 weeks or 43-45 weeks were obtained. Protein carbonylation was evidenced in uterine epithelium by fluorescent immunohistochemistry techniques. Reproductive outcome was evaluated by counting the number of viable fetus. H&E staining of uteroplacental compartment was used for histological examination. SOD activity and expression were determined in placental bed by spectrophotometry and western blotting, respectively. Aged females were treated, prior to and during pregnancy, with a SOD mimetic (TEMPOL, 1 mM) or a NOX inhibitor (apocynin, 5 mM) and reproductive outcome and uteroplacental histology were re-evaluated. Results are presented as mean ± SEM. Statistical analysis was performed with Student's t test.

Results: The uterus of non-pregnant aged mice was hypertrophied and contained cysts, when compared to young mice. Pregnant aged females showed an age-related decrease in the number of viable fetuses [young females 7.4 ± 0.6 (n=9) and aged females 2.2 ± 0.5 (n=13), P<0.0001]. Moreover, this decrease was accompanied by a significant reduction of the uterine decidua thickness [young females, 695 µm ± 49 (n=6) and aged females, 411 µm ± 45 (n=4), P<0.005]. Moreover, protein carbonylation was increased. Total SOD activity in placental bed did not change with increased reproductive age. Although a significant increase was observed when MnSOD activity was subtracted from total SOD [in U SOD/mg protein: young females 1.0 ± 0.4 (n=5) and aged females 2.8 ± 0.5 (n=6) P<0.05] no changes were observed in Cu/Zn SOD expression. Supplementing aged female diet with apocynin, but not TEMPOL, increased the number of viable fetuses [control 2.2 ± 0.5 (n=13) and apocynin 3.8 ± 0.7 (n=12), P<0.05]. Most impressively it also increased the height of the decidua layer (in µm: control 411 ± 45 (n=4) and 796 ± 142 (n=4), P<0.05). Regarding fetuses weight apocynin decreased this parameter [in mg: control 1.3 ± 0.1 (n=30) and apocynin 1.1 ± 0.1 (n=28), P<0.05] and TEMPOL improved it [in mg: control 1.30± 0.1 (n=30) and TEMPOL 1.5 ± 0.04 (n=18), P<0.05].

Conclusion: The present study provides evidence that alterations in uterine redox balance play a fundamental role in the correct development of uterine decidua and female reproductive capacity.

Relevance: This work draws the attention to the fact that specific antioxidant molecules modulate different cell pathways important for correct implantation and embryo development.

CANCER | POSTER 09

Clasp1 is essential for neonatal lung function and survival

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CLIP-Associating Proteins (CLASPs) are microtubule plus-end-tracking proteins with an important role in the regulation of microtubule dynamics and involved in chromosome segregation during mitosis. In *Drosophila* and yeast, CLASP loss-of-function resulted in the generation of mitotic abnormalities, with subsequent aneuploidy, indicating that this protein is essential for cell division and viability. In *C.elegans*, RNAi of the orthologue *cls*-2 yielded embryonic lethality. In mammals, two paralogues, CLASP1 and CLASP2 exist, which prompted us to further investigate their individual roles. For that, we successfully generated Clasp1 knockout (KO) mice through homologous recombination in embryonic stem cells. Our data demonstrate that targeted disruption of *clasp1* not only causes intrauterine growth restriction, as revealed by low body weight at birth, but also leads to early neonatal lethality as a consequence of an acute respiratory failure, with the KO animals displaying cyanotic features shortly after being born. Histological analyses of KO animals prove lack of inflation in the lungs without any obstruction of the respiratory tract being observed. Our immunohistochemical results suggest a delayed fetal lung maturation characterized by decreased sacculation that, however, does not impair the presence of surfactant. Importantly, protein expression results show Clasp1 presence in the lungs throughout embryonic development. Altogether, our findings reveal a novel and critical role for the plus-end tracking protein Clasp1 in fetal growth, as well as proper lung development and respiratory function in a mammalian model system.

An Interferon-γ-delivery system based on chitosan/poly(γ-glutamic acid) polyelectrolyte complexes modulates macrophage phenotype

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Macrophages represent a large component of the tumour microenvironment and are described to establish interactions with cancer cells, playing crucial roles in several stages of cancer progression. The functional plasticity of macrophages upon stimulation from the environment makes them susceptible to the influence of cancer cells but also points them as promising therapeutic targets.

In this report, we describe a drug delivery system to modulate macrophages from the pro-tumour M2 towards the anti-tumour M1 phenotype, based on the incorporation of a pro-inflammatory cytokine (Interferon-γ) in Chitosan (Ch)/Poly(γ-glutamic acid) (γ-PGA) complexes. Ch is a biocompatible cationic polysaccharide extensively studied and γ-PGA is a poly-amino acid, biodegradable, hydrophilic and negatively charged. These components interact electrostatically, due to opposite charges, resulting in self-assembled structures that can be modulated to carry and deliver active molecules such as drugs, proteins and others.

Ch and γ-PGA were self-assembled into polyelectrolyte multilayer films (PEMs) of 371 nm thickness, using the layer-by-layer method. Interferon-γ (IFN-γ) was incorporated within the Ch layers at 100 and 500 ng/mL. Ch/γ-PGA PEMs with IFN-γ were able to modulate the phenotype of IL-10-treated macrophages at the cell cytoskeleton and cytokine profile levels, inducing an increase of IL-6 and a decrease of IL-10 production. Additionally, the pro-invasive role of IL-10-treated macrophages was hindered, as their stimulation of gastric cancer cell invasion decreased from 4 fold to 2 fold, upon modulation by Ch/γ-PGA PEMs with IFN-γ. Based on these findings, we hypothesize that the controlled release of IFN-γ at the tumour site would critically increase its therapeutic efficacy in reversing the phenotype of tumour-associated macrophages. This is the first report proposing Ch/γ-PGA PEMs as IFN-γ delivery systems with the aim of modulating macrophage phenotype, and thus counteracting their stimulating role on gastric cancer cell invasion.

CANCER | POSTER 11

Matrix metalloproteases as maestros for the dual role of LPS- and IL-10-stimulated macrophages in cancer cell behaviour

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The interactions established between macrophages and cancer cells are largely dependent on instructions from the tumour microenvironment. Macrophages may differentiate into populations with distinct inflammatory profiles, but knowledge on their role on cancer cell activities is still very scarce. In this work, we investigated the influence of pro-inflammatory (LPS-stimulated) and anti-inflammatory (IL-10-stimulated) macrophages on gastric and colorectal cancer cell invasion, motility/migration, angiogenesis and proteolysis, and determined the associated molecular mechanisms. Our results show that IL-10-stimulated macrophages are more efficient in promoting *in vitro* cancer cell invasion and migration. In addition, soluble factors produced by these macrophages enhanced *in vivo* cancer cell-induced angiogenesis, as opposed to their LPS-stimulated counterparts. We further demonstrate that differences in the ability of these macrophage populations to stimulate invasion or angiogenesis cannot be explained by the EGFR-mediated signalling, since both LPS- and IL-10-stimulated macrophages similarly induce the phosphorylation of cancer cell EGFR, c-Src, Akt, ERK1/2, and p38. Interestingly, both populations exert distinct proteolytic activities, being the IL-10-stimulated macrophages the most efficient in inducing matrix metalloprotease (MMP)-2 and MMP-9 activities. Using a broad-spectrum MMP inhibitor, we demonstrate that proteolysis was essential for macrophage-mediated cancer cell invasion and angiogenesis. Therefore, we propose that IL-10- and LPS-stimulated macrophages distinctly modulate gastric and colorectal cancer cell behaviour, as result of distinct proteolytic profiles that impact cell invasion and angiogenesis.

CANCER | POSTER 12

P-cadherin expression modifies the biomechanical properties of breast cancer cells and promotes increased protrusive activity in 3D cell culture models

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Physical forces are involved in several cellular processes during development and homeostasis, as well as in pathological conditions. Cell-cell adhesion molecules, namely E-cadherin and P-cadherin, have a crucial role in the maintenance of normal epithelia architecture. However, molecular alterations in the mechanical balance mediated by their adhesive forces are known to promote tumorigenesis. In epithelial cancers, E-cadherin acts as an invasion suppressor protein, being lost in highly infiltrative tumours; in contrast, P-cadherin is overexpressed in high-grade tumours, being associated with poor patient survival. *In vitro*, P-cadherin promotes cell invasion by inhibiting the E-cadherin suppressive function.

Although the functional effects of P-cadherin in tumour progression have been already explored, nothing is known concerning how its expression affects the biomechanical properties of cancer cells. To achieve that, two different breast cancer cell models were used, where P-cadherin expression was manipulated (MCF-7/AZ and BT20).

Using atomic force microscopy (AFM), we found that P-cadherin-overexpressing cells significantly present a higher area and volume, as well as a decreased height. These cells also showed higher cell elasticity. Further, we still found that P-cadherin-overexpressing cells were easier to separate than control cells, meaning that these are softer and more deformable, but also that cell-cell adhesion is clearly compromised. These observations were further confirmed by software analysis of confocal images.

Invasive cell behaviour in 3D *in vitro* cultures was also analysed by time-lapse microscopy, by embedding compact cell aggregates in collagen and matrigel extracellular matrixes (ECM). P-cadherin-overexpressing cells showed a significant higher number of protrusions, as well as increased protrusion's lenght in collagen, with very low or no protrusive activity in matrigel.

In conclusion, P-cadherin induces morphological and biomechanical cell alterations that are usually associated to more invasive cancer cells. Although preliminary, the results anticipate an important role for P-cadherin in mechanotransduction signalling in breast cancer cells.

CDH3/P-cadherin is negatively regulated by TAp63 in a p53-dependent manner in breast cancer cells. Effects on P-cadherin-mediated invasion and self-renewal.

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Introduction: P-cadherin, a cell-cell adhesion molecule codified by the *CDH3* gene, is frequently expressed in high-grade breast cancer, being a well-established indicator of poor patient prognosis and has been reported as an important inducer of cancer cell migration and invasion. P-cadherin also confers stem cell features to breast tumorigenic cells that could be linked to the aggressive behaviour of basal-like breast cancers. P-cadherin has been associated with already described stem cell markers, such as p63, which was recently demonstrated to transcriptionally regulate *CDH3* in a context of the developmental biology. In fact, the parallelism between p63 and P-cadherin interestingly involves the cancer and the developmental setting. In cancer, however, the relationship between p63 and P-cadherin was only explored in a pathological perspective.

Material and Methods: Breast cancer cell lines and luciferase report assays were used to demonstrate the transcriptional regulatory effect of p63 isoforms on CDH3 promoter. *In vitro* functional assays were used to demonstrate the impact of this transcription factor in P-cadherin-mediated effects.

Results and Discussion: We demonstrate that TAp63 isoforms transcriptionally represses *CDH3* promoter, downregulating P-cadherin protein expression in MCF7 breast cancer cells. This repression is functionally reflected on P-cadherin-induced breast cancer cellular invasion and mammosphere-forming efficiency. Interestingly, we also observed that this effect of TAp63 isoform on *CDH3*/P-cadherin was not replicated in cells harbouring p53 mutations, and that the induction of p53 hotspot mutations on p53 wild-type cells restored *CDH3* promoter activation. These results suggest that the repressive effect of TAP63γ isoform onto *CDH3* promoter is disabled by the p53 mutants. The validation of these observations in human breast cancer samples revealed that that breast tumours expressing TAp63γ isoform, but harbouring some type of known pathogenic p53 mutations were positive for P-cadherin expression, while the only case negative for P-cadherin expression was the one where no p53 mutations were detected.

Conclusion: Taken together, our data reveal previously unknown molecular functions of TAp63γ isoforms on *CDH3*/P-cadherin where TAp63γ is able to represses *CDH3* promoter activity and P-cadherin expression levels, being this regulation dependent of p53 mutational status.

Relevance of MUC1 splice variants in pancreatic carcinogenesis

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MUC1 is a heavily glycosylated transmembrane glycoprotein expressed in low levels in apical surfaces of epithelial cells and overexpressed in more than 80% of pancreatic tumors. This overexpression is well known to be correlated with tumor initiation, tumor progression and poor survival of cancer patients. The MUC1 gene encodes a protein with a large extracellular domain with a tandem repeat region, a self-cleaving domain, a transmembrane domain and a highly conserved cytoplasmic domain (MUC1-CD) that participates in several oncogenic signaling pathways. Different MUC1 isoforms, generated by alternative splicing events, have been associated with carcinogenesis processes. MUC1/Y, a splice variant that lacks the tandem repeat region was associated with breast cancer and together with similar isoforms (MUC1/X and MUC1/Z) was also associated with malignant ovarian tumors. In contrast, the MUC1/SEC isoform, which lacks the cytoplasmic tail and the transmembrane domain was associated with absence of malignancy. Despite a few reports, the functional significance of the MUC1 splice variants, is not well known.

The main objective of this work is to study the relevance of MUC1 splice variants in pancreatic carcinogenesis. We used two *in vitro* models established from human pancreatic duct cells (hTERT-HPNE and hTERT-THPNE cell lines) to reproduce the early stages of pancreatic carcinogenesis. We used a lentiviral system to transduce two different MUC1 splice variants (MUC1/X and MUC1/S2) into both cell lines. A detailed evaluation of MUC1 isoforms overexpression impact in phenotypic characteristics (morphology, proliferation, aggregation, migration and invasion) and in the associated oncogenic signaling pathways is currently being performed.

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The mechanisms underlying the regulation of normal breast epithelial architecture mediated by P-cadherin expression

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P-cadherin has important tumour promoting properties in breast cancer. However, in the normal breast, P-cadherin behaves as a classic cell-cell adhesion molecule, being important for the maintenance of normal tissue architecture, especially in the myoepithelial cell compartment (1). Studies in P-cadherin knockout mice have shown that loss of P-cadherin causes hyperplasia and dysplasia (2), as well as precocious branching morphogenesis of the mammary gland (3); though, the specific mechanisms underlying the regulation of normal breast epithelial architecture mediated by P-cadherin expression have not been explored.

Using 2D and 3D cultures of normal breast cell lines (MCF10A and 226L), we have found that P-cadherin expression is implicated in the control of cell-cell and cell-matrix adhesion, as well as with epithelial cell polarity. Both MCF10A and 226L cells form spherical and compact colonies in 3D matrigel cultures. However, P-cadherin knock-down decreased the number and the size of the organoids that were formed by these cells. Our data still showed that P-cadherin silencing reduces Zona 0ccludens (ZO)-1 and ZO-2 expression, as well as Claudin-3 expression. Additionally, we found that P-cadherin inhibition decreased pAKt and pSrc signaling as well as a6b4 integrin expression, an heterodimer that is crucial for the interaction of the normal epithelial cells with the basement membrane components. In fact, we found that a6 integrin knock-down caused acini disorganization in 3D matrigel. Analysis of the polarity determinants Par3, aPKC and Scribble showed no difference in the expression of after P-cadherin inhibition. Furthermore, preliminary data show that there is a link between P-cadherin expression and the microtubule (MT) network, since the treatment with MT perturbing drugs (nocodazole or taxol) significantly increased P-cadherin and Claudin 3 expression in MCF10A cells. Further studies are needed to clarify the link between P-cadherin/tight junctions/a6b4 integrin/basement membrane, and possibly the MT network, in determining epithelial cell polarity and tissue architecture of normal breast. Understanding the role of P-cadherin expression in the basal layer of the normal breast may help clarify the progression of pre-neoplastic lesions, such as hyperplasia, as well as the alterations in the stem cell niche that may lead to the precocious differentiation of the mammary gland in the P-cadherin knockout mice.

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P-cadherin expression: its role in the regulation of metabolic properties of breast cancer stem cells

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Introduction: Constitutive upregulation of glycolysis is likely to be a cellular adaption to hypoxic conditions, being recently recognized as a hallmark of cancer [1, 2]. In fact, hypoxia and consequent metabolic alterations have already been implicated in breast cancer progression [3]; additionally, it has been demonstrated that breast cancer stem cells use preferentially glycolysis over oxidative phosphorylation as their main source of energy [4], which make them resistant to hypoxia, as well as protect them from damage induced by mitochondrial-produced ROS (reactive oxygen species).

P-cadherin, a biomarker of basal-like breast cancer and a poor prognostic factor in this disease [5], mediates stem-like properties, as well as resistance to radiation therapy [6]. Moreover, we have previously demonstrated that P-cadherin aberrant expression is associated with hypoxic, glycolytic and acidosis markers in breast carcinomas, that HIF-1α stabilization increases membrane P-cadherin expression and that P-cadherin enriched cell populations show increased GLUT1 and CAIX expression. These populations also comprise high mammosphere forming efficiency, suggesting that P-cadherin overexpressing breast cancer cells are more likely to exhibit increased glycolysis and to survive to metabolic-driven pH alterations [7]. In this work, our aim was to understand if P-cadherin expression could be regulating breast cancer stem cell's metabolic properties, which may explain its association with radiotherapy resistance and tumor aggressiveness.

Materials and methods: Using a siRNA-mediated approach, we silenced the expression of P-cadherin in breast cancer cells and evaluated the OCR/ECAR rate as a measure of OXPHOS and glycolysis, using the SEAHORSE technology. ATP measurements were performed using CellTiter-Glo Luminescent Cell Viability Assay kit and ROS content was evaluated by luminescence after incubation with DCHF-DA probe. Western blot and zymography was used to evaluate the state of ROS scavenging systems.

Results and Discussion: We found, for the first time, that P-cadherin silencing was able to modulate cellular bioenergetics of breast cancer cells by allowing an increased OCR/ECAR rate and cellular ATP content. Furthermore, we demonstrated that P-cadherin expression is associated with the production of low ROS levels, by inducing the upregulation of ROS scavenging systems, such as SOD1 and SOD2 (superoxide dismutase 1 and 2). Although preliminary, we believe that the obtained data is demonstrating that P-cadherin might be a player of therapeutic resistance through the metabolic regulation of breast cancer stem cells.

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CANCER | POSTER 17

Heteroplasmic germinal and somatic mtDNA mutations evaluated in NGS data – implications in cancer

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The majority of the mitochondrial DNA (mtDNA) mutations implicated in many complex diseases are heteroplasmic (coexistence of variants for the same SNP in the same mitochondrion or cell), as usually they consist in *de novo* mutations, which are in the process of becoming fixed or getting lost. This behavior affects both germinal mutations and somatic mutations, the last ones eventually appearing in cancer cells. Next-generation sequencing is enabling to evaluate heteroplasmy with a high precision, making it possible to detect levels as low as 1%.

Here we analysed 124 mother-child pairs from five populations (21 African, 32 European, 15 South Asians, 30 East Asians and 26 Americans) sequenced by the company Complete Genomics, and made available in the 1000 Genomes database. High quality (Phred quality score above 20) mapped mtDNA sequences were extracted from the Sequence Read Archive (SRA) and the ones that aligned exclusively in the mtDNA genome were kept (filtering of NUMTs). A mutation was considered heteroplasmic after passing a stringent log likelihood ratio. For comparison, we collected a dataset of somatic mtDNA mutations in cancer patients available in the literature, and a dataset of polymorphisms in the population (healthy adults) deposited in GenBank.

De novo mutations in children behave similarly to the somatic cancer dataset in terms of potential pathogenic variants in mtDNA coded proteins, as expected in the absence of purifying selection. This pattern is distinct from the database of polymorphisms observed in the healthy adult population, which displays a significantly lower proportion of potential pathogenic mutations. The mutations that are lost in the transmission from mother to child also present a high pathogenic score, while the heteroplasmic mutations that are shared between the pair present a distribution of pathogenic scores similar to the adult human population. So, purifying selection acts already in the scale of one generation, rendering the highly pathogenic mutations rare even within a family (the effect is of course stronger at the level of a population – no highly pathogenic mutation is shared between many individuals). But the random occurrence of the novo mutations reintroduces the pathogenic mutation in the population. The understanding of this dynamic in the germinal transmission can help in evaluating the pathogenic potential of mtDNA mutations in cancer, which not being primary oncocytic event, can lead nevertheless to a worse phenotype or interfere with response to treatment.

CANCER | POSTER 18

Expression of ST3GAL4 leads to SLeX expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells

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Sialyl-Lewis X (SLe^X) is a sialylated glycan antigen frequently expressed on the cell surface during malignant cell transformation and is associated with cancer progression, aggressiveness and poor prognosis for the patients. Increased expression of SLe^X antigen in cancer has been associated to alterations in the expression of sialyltransferases, though the exact molecular mechanism has not been completely understood.

In this study we evaluated the capacity of two sialyltransferases to synthesize SLe^X antigens in gastric carcinoma, by overexpressing ST3GAL3 and ST3GAL4 in MKN45 cells. In addition, we determined the role of SLe^X expression in gastric cancer cell behavior both *in vitro* and *in vivo* using the chicken chorioallantoic membrane (CAM) model. Furthermore, we evaluated the activation of tyrosine kinase receptors and downstream molecular targets and described a possible molecular signaling mechanism involved in SLe^X-induced behavior.

Our results showed that the expression of ST3GAL4 in MKN45 gastric cancer cells leads to the synthesis of SLe^X antigens and to an increased invasive phenotype both *in vitro* and in the *in vivo* CAM model. Analysis of phosphorylation of tyrosine kinase receptors showed a specific increase in c-Met activation. The characterization of downstream molecular targets of c-Met activation, involved in the invasive phenotype, revealed increased phosphorylation of FAK and Src proteins and activation of Cdc42, Rac1 and RhoA GTPases. The SLe^X-induced invasive phenotype was associated to c-Met signaling since inhibition of c-Met and Src activation abolished the observed increased cell invasive phenotype.

In conclusion, the overexpression of ST3GAL4 leads to SLe^X antigen expression in gastric cancer cells and induces an increased invasive phenotype through the activation of c-Met and downstream signaling activation.

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CANCER | POSTER 19

Genetic variants in the *IL1A* gene region contribute to intestinal-type gastric carcinoma susceptibility in European populations

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The most studied genetic susceptibility factors involved in gastric carcinoma (GC) risk are polymorphisms in the inflammation-linked genes interleukin 1 (*IL1*) B and *IL1RN*. Despite the evidence pointing to the *IL1* region, definite functional variants reproducible across populations of different genetic background have not been discovered so far. A high density linkage disequilibrium (LD) map of the *IL1* gene cluster was established using HapMap to identify haplotype tagSNPs. 87 SNPs were genotyped in a Portuguese case-control study (358 cases, 1485 controls) for the discovery analysis. A replication study, including a subset of those tagSNPs (43), was performed in an independent analysis (EPIC-EurGast) containing individuals from 10 European countries (365 cases, 1284 controls). Single SNP and haplotype block associations were determined for GC overall and anatomopathological subtypes. The most robust association was observed for SNP rs17042407, 16Kb upstream of the *IL1A* gene. Although several other SNP associations were observed, only the inverse association of rs17042407 allele C with GC of the intestinal type was observed in both studies, retaining significance after multiple testing correction (p=0.0042) in the combined analysis. The haplotype analysis of the *IL1A* LD block in the combined dataset revealed the association between a common haplotype carrying the rs17042407 variant and GC, particularly of the intestinal type ($p=3.1x10^{-5}$) and non cardia localisation ($p=4.6x10^{-3}$). These results confirm the association of IL1 gene variants with GC and reveal a novel SNP and haplotypes in the *IL1A* region associated with intestinal type GC in European populations.

CANCER | POSTER 20

Differences in gastric microbiota in gastritis and gastric carcinoma

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We tested the hypothesis that bacteria other than *Helicobacter pylori* exist in the stomach and that an altered gastric microbiota is present in gastric carcinoma.

We studied 25 gastric carcinoma cases and 73 chronic superficial gastritis control subjects. 16S rRNA bacterial genes were amplified in gastric biopsy specimens using primers targeting the V5-V6 regions, sequenced by the Ion PGM sequencing platform, and aligned for taxonomic classification to fully sequenced bacterial genomes using the QIIME pipeline.

From the 98 gastric samples, a total of 13.108.150 16S rRNA gene filtered sequences were obtained. The comparison of gastric carcinoma and gastritis cases for the presence and relative abundance of taxa showed that carcinoma cases had an enrichment of the phylum Proteobacteria (77% vs 55% relative abundance for carcinoma and gastritis cases, respectively) and depletion of Bacteroidetes and Fusobacteria (3% vs 14% and 0.1% vs 2%, respectively). Within Proteobacteria, the relative enrichment was most noticed for the families Xanthomonadaceae (19% vs 0.3%; $p < 0.001$) and Alcaligenaceae (16% vs 3% $p < 0.001$).

Our findings that there is an altered stomach bacterial community associated with gastric carcinoma may impact cancer prevention and treatment strategies of patients with precancerous disease. These results may provide a new interpretative frame for the incomplete efficacy of successful eradication of *H. pylori* in preventing gastric carcinoma. Further studies are needed to evaluate the clinical impact of these findings.

Anti-Cancer Activity of New Ionic Liquids or salts from Beta-Lactam Antibiotics

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Ionic liquids (ILs), in recent years, have been a leading topic in science and technology. The first ILs were used mainly because of their unique physical and chemical properties. Nowadays, most of the potential applications are now associated to their biological properties. ILs are being applied in pharmaceutical industry to solve problems like the low solubility and thus bioavailability of pharmaceutical compounds, or the polymorphism problem[1]. Our group is developing several ILs based on Active Pharmaceutical Ingredients (APIs).[2-4]

Our main goal is the study of the anti-cancer properties of new ILs from beta-lactam antibiotics. This approach is of huge interest in pharmaceutical industry as cation and anion composition of ILs can greatly alter their desired properties[1, 4, 5].

The cations hydroxide used in this work, were obtained on Amberlite resin (in the OH form) in order to exchange halides[4]. These new synthesized ILs were then used in order to evaluate their anti-proliferative activity, by colorimetric assays, against some human cancer cell lines and primary human cell lines.

The results show that the novel beta-lactam salts (as ILs or molten salts) presented low values of IC50 and LD50 against cancer cell lines, showing that they have great potential regarding their use against cancer cell lines. Also, it was observed low cytotoxicity against primary cell lines which might indicate that these compounds are not toxic to human cell lines.

In conclusion it is relevant to highlight that the right selection of the counter-ions can explained these antitumoral activities due the stronger and more stable cation-anion interactions. The adequate organic cations can establish efficient ion pairs acting as lipophilic drug carriers as well as improving the water solubility, permeability and bioavailability of final pharmaceutical salts.

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CANCER | POSTER 22

Polar ejection forces are involved in Spindle Assembly Checkpoint satisfaction during mitosis of unreplicated genome

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Complete DNA replication during S phase and establishment of bipolar attachments during mitosis is from crucial importance for the life of the cell. In our work we are investigating the Spindle Assembly Checkpoint (SAC) satisfaction in the absence of interkinetochore tension. For that propose, we used RNAi depletion of Dup (double parked), a protein involved in origin of replication and mitotic checkpoint, which allowed *Drosophila* S2 cells to enter mitosis without previous replication of the genome. This interesting phenomenon is known as mitosis of an unreplicated genome (MUG). S2 cells undergoing MUG have condensed single chromatids, unstably attached to spindle microtubules. By using live cell imaging and *Drosophila* S2 cells stably expressing fluorescently labeled proteins we were able to follow the dynamics of mitotic proteins during MUG. *Drosophila* S2 cells undergoing MUG stayed longer time in mitosis compared with control cells, which was result of slower cyclin B degradation in RNAi treated cells. Nevertheless, after delay (2-3h) Dup depleted cells were able to exit mitosis previously satisfying the SAC.

The correct mechanism how SAC can be satisfied in the absence of interkinetochore tension is unknown. Here we show the role of PEFs in establishment of intrakinetochore stretch, as a mechanism for SAC satisfaction in the absence of interkinetochore tension during mitosis of unreplicated genome.

Evaluation of the biological impact of simple O-glycans in gastric cancer applying glycoengineered simplecell lines strategy

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Altered glycosylation is a common feature of cancer and aberrant expression of sialylated structures, such as sialyl-Tn (STn), at the surface of cancer cells has been extensively reported and correlated with an invasive phenotype in various cancer models [1-3]. In fact, STn expression has been shown to be an independent indicator of poor prognosis in gastric cancer [4].

Our aim is to study the biological impact of simple O-glycans GalNAc (Tn) and NeuAc-GalNAc (STn) expression in gastric cancer cell lines, using as strategy the ZFN targeting of the core-1 synthesis step by knockout of COSMC.

AGS and MKN45 gastric cancer glycoengineered simplecell (AGS SC and MKN45 SC) lines, recently established by our group in collaboration with the Copenhagen Center for Glycomics (Campos *et al*, submitted), were used to study the biological impact associated with an increase expression of truncated O-glycans (Tn and STn) at cell surface in the biology of tumour cells. We evaluated morphologic aspects, migration and invasion *in vitro* as well as angiogenesis and tumourigenesis using *in vivo* chorioallantoic membrane (CAM) and nude mice models, respectively.

Glycoengineered simple cell lines showed major cell morphology alterations and enhanced capacity to migrate and to invade *in vitro* comparatively to the wildtype cell lines. These cells expressing simple O-glycans showed altered capacity to induce vessel formation in the CAM model and produced smaller tumours after cell injection in nude mice, although these cells had a bigger impact in the mice survival comparatively to the control cells.

COSMC knockout cell lines expressing high levels of Sialyl-Tn glycoforms revealed to behave more aggressively than the wildtype cells, suggesting that STn has a crucial impact in key signaling pathways controlling epithelial cell behavior, mediating cell motility, invasion, and therefore modulate tumour cell aggressiveness.

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Establishment and characterization of a CD44v6^{+} gastric cancer cell line: a model for nanoparticle based, early diagnosis of gastric cancer lesions

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Introduction: Although gastric cancer (GC) incidence has been diminishing in the past few years, it still features as the fourth most common neoplastic malignancy and has the third highest mortality rate of cancer associated malignancies. Early diagnosis of the disease is essential to improve early detection and tumor prognosis of individuals with gastric cancer (GC), or at risk of developing the disease. Recently our group has identified CD44v6, an alternative transcript of the CD44 protein, as an adequate molecular marker for GC and corresponding pre-malignant lesions. Identifying molecular probes able to specifically target CD44v6 in GC invasive cells, which can then be grafted in nanoparticles ultimately designed to attach to the stomach wall, cross the mucosal barrier epithelium, target CD44v6 positive cells and provide a signal for diagnosis, can be a valuable tool to improve early diagnosis of the disease.

Here we describe the generation and characterization of a GC derived cell line expressing *de novo* the surface protein CD44v6 or its canonical form, CD44std, which will be used to test the efficacy of the functionalized nanoparticles.

Materials and Methods: The sequences encoding the CD44 standard (CD44-03 - ENST00000263398) and CD44v6 (variant CD44-04 - ENST00000415148) were cloned unto a pIRES2-EGFP expression vector and the human GC derived cell line MKN-28, was transfected with these expression vectors.

A functional study to assess if *de-novo* expression of CD44v6 or CD44std has a relevant role in cancer cell behavior was undertaken. Invasion, migration and slow aggregation assays were performed. Resistance to apoptosis was also tested by challenging cells with serum starvation or the drug Cisplatin. The expression of downstream effectors/interactors of CD44v6 was also analyzed.

Results and discussion: We have successfully established a GC derived cell line expressing *de-novo* the CD44v6 variant or its canonical form, CD444std. Both cell lines were characterized functionally by studying migration, invasion and aggregation ability. Expression of the CD44 isoforms does not seem to alter functional behavior of the cell lines. However, expression of these proteins seems to confer resistance to apoptosis induced by cisplatin treatment, which may be correlated with an increase in the expression of downstream effectors of CD44. Expression of EGFR, ERK1/2, Akt, c-Met and p38 was assessed. p38 MAPK, seems to be up-regulated in the cell lines expressing the CD44 isoforms as well as in response to cisplatin treatment. Notably, *in vivo* inhibition of p38 has been shown before to increase the sensitivity of GC tumor cells to cisplatin, together with the downregulation of multidrug resistance (MDR)-1, a marker of resistance to chemotherapy.

Our results suggest that de novo expression of CD44 isoforms, may confer increased resistance of GC cells to apoptosis. This mechanism seems to be mediated in part by the expression of higher levels of p38 MAPK.

A co-culture of MKN-28 CD44($^{+}$) and CD44($^{-}$) cells will later be used to assess the specificity of the functionalized probes currently under development.

TXA1.HCl induces autophagy in NCI-H460 cells and reduces the growth of these lung cancer cells xenografted in nude mice

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Modulation of autophagy has been emerging as a potential therapeutic strategy in many diseases, including cancer [1]. Our previous work has identified a hit molecule, named TXA1, from the CEQUIMED-UP library which is a potent inducer of autophagy in a melanoma cell line (submitted for publication). The soluble version of this molecule, TXA1.HCl, was also shown to induce autophagy in other cancer cell lines (work in preparation).

The present work aimed at: i) understanding the mechanism of action of TXA1.HCl in a lung cancer cell line; and ii) investigating its *in vivo* toxicity and the antitumour activity in xenographs of a human tumour lung cancer cell line in nude mice.

TXA1.HCl decreased NCI-H460 viable cell number and modulated cellular autophagy. Results from gene expression analysis with DNA microarrays of cells treated with of this molecule indicated that the compound induced significant alterations in the expression of genes involved in lipid metabolism. These results are currently being confirmed by qRT-PCR. Interestingly, autophagy has already been associated with the regulation of lipid metabolism in cancer [2, 3]. Importantly, sub-cutaneous injection of this compound into nude mice did not cause toxicity to mice and significantly decreased tumour growth of NCI-H460 cells xenografts.

In conclusion, this ongoing work shows the potential of this hit molecule as a modulator of autophagy, and possibly of lipid metabolism, in tumour cells. Future work will allow confirming the mechanism of action by studying the tumours removed from the treated mice in comparison with the non-treated controls.

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Searching for the molecular mechanism by which P-cadherin expression confers resistance to DNA damage in normal and breast cancer cell lines

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P-cadherin is a breast cancer poor prognostic factor and a cancer stem cell biomarker, with direct relevance in the stem cell activity of basal-like breast cancer. Importantly, we have also found that P-cadherin expression confers resistance to X-ray-induced cell death; however, the mechanisms involved in this resistance are not yet clarified (Vieira *et al.*, 2012).

In an attempt to unravel the role of P-cadherin in DNA damage resistance, we performed the downregulation of this protein using siRNA in MCF10A (normal-like) cells and BT-20 (breast cancer) cells, when these were exposed to different DNA stress-inducing stimuli, namely hydrogen peroxide (H2O2), UV-C light (UV) and Taxol. The mammosphere assay was performed, in order to study the impact of concomitant DNA damage and P-cadherin in cancer stem cell activity. The DNA-damage response/repair pathway (DDR/R) was analyzed through the the expression of ATM, BRCA1, Chk1 and Chk2 proteins by western blot. DNA breaks were evaluated by the gamma-H2AX immufluorescence staining, as well as by the alkaline single cell gel electrophoresis assay (comet assay). To further confirm the role of P-cadherin in cell death resistance, the PI / Annexin V assay was also performed.

Both cell lines were very sensitive to UV irradiation exhibiting elevated cell death. The treatments with H202 and Taxol showed a significant reduction in the stem cell population which, after P-cadherin knock-down, showed a tendency to decrease further in both cell lines. As expected, we found that H202, UV light and - to a lower extent - Taxol were able to induce several players of the DDR/R pathway. Interestingly, P-cadherin inhibition had also an impact in the expression of these molecules. Accordingly, the three DNA stress-inducing agents increased DNA damage, which was further increased by P-cadherin silencing in both cell lines MCF10A and BT-20. Cell death was increased by P-cadherin silencing in the presence of H202 and Taxol in both cell lines MCF10A and BT-20 and in the absence of any stimuli in the normal-like cell line - MCF10A.

Thus, although preliminary, or data show for the first time that P-cadherin has a role in the DDR/R pathway, promoting DNA damage resistance and, consequently, cell death resistance. Although further experiments need to be performed, our work points to the idea that breast cancer patients undergoing radiation therapy could potentially benefit from a P-cadherin inhibition strategy.

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Studying *Ganoderma lucidum* as a source of molecular inducers of autophagy

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Autophagy is one of the three main mechanisms of programmed cell death[1]. One of the basis of cancer therapy is to induce death of tumour cells. There are several clinically approved molecules that induce cell death[2,3], but unfortunately not all tumours highly resistant to cell death respond to the existing therapies. Therefore, the search for new molecules capable of inducing tumour cell death is an area of growing interest. In this context, modulators of autophagy have become very attractive in recent years. Previous work performed by some of us has shown that methanolic extracts of the medicinal mushroom *Ganoderma lucidum* were modulators of cellular autophagy[4]. The aim of the present work was to further understand the cellular mechanisms involved in the autophagy modulation and to identify bioactive compounds responsible for this modulation.

A methanolic extract obtained by cold extraction (-20°C) from *G. lucidum* was studied with respect to its ability to induce autophagy in a gastric adenocarcinoma cell line (AGS). The presence of autophagic vacuoles was observed following transfection of cells with a mCherry-LC3 expression vector and the levels of some autophagic proteins were analysed by Western Blot. Cells were also treated with the lysossomal protease inhibitors E-64d/pepstatin together with the extract, in order to confirm if the extract induced autophagy or a decrease in the autophagic flux. The levels of the autophagic proteins after this treatment were also evaluated by Western Blot.

An increase in the autophagic vacuoles was observed in cells treated with the extract (concentrations corresponding to the GI_{50} and $2\times GI_{50}$) 24h before transfection with the mCherry-LC3 vector. In addition, treatment of cells with the same concentrations of the extract for 48h induced an increase in the expression of LC3-II together with a slight reduction in the levels of p62, particularly with the highest concentration. Additionally, cells treated with the extract together with E-64d/pepstatin expressed higher levels of LC3-II and p62, when compared with cells treated only with the extract, indicating that *G. lucidum* caused an induction of autophagy rather than a decrease in the autophagic flux.

With these results we can conclude that the methanolic extract from the mushroom *G. lucidum* may be a source of compounds capable of inducing autophagy. Further studies to identify the bioactive compounds present in the tested extract and responsible for such activity are still ongoing.

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TERT promoter mutations: a possible worse prognosis marker in cutaneous melanoma

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Introduction: The reactivation or re-expression of telomerase (TERT) is a widespread feature of neoplasms. *TERT* promoter mutations were recently reported in cutaneous melanoma and were advanced to result from UV-radiation.

This study aimed to evaluate the role of the presence of *TERT* promoter mutation in the etiopathogenesis of melanoma.

Material and Methods: We assessed *TERT* promoter mutations in 4 cutaneous melanoma cell lines, 21 nevi, 116 primary cutaneous melanomas, 6 uveal melanoma cell lines and 26 primary ocular melanomas (21 uveal melanomas and 5 conjunctival melanomas). In all of the aforementioned samples we had previously obtained data of the *BRAF* status. We examined the TERT protein expression by immunohistochemistry in cutaneous melanomas. We looked at the relationship between the presence of *TERT* promoter mutation and the immunohistochemical data, and explore the putative association between *TERT* promoter mutation and the clinico-pathological and prognostic parameters in cutaneous melanoma.

Results and Discussion: *TERT* promoter mutations were detected in the 4 cutaneous melanoma cell lines analyzed and in 22% of the 116 cutaneous melanomas. No alterations were found in nevi, uveal melanoma cell lines and ocular melanomas. In cutaneous melanomas, *TERT* promoter mutations were generally restricted to intermittent sun-exposed areas and significantly associated with nodular and superficial spreading subtypes, increased thickness, ulceration, increased mitotic rate, shorter disease-free and overall survival. Moreover, *TERT* promoter mutations significantly associated with the presence of BRAFV600E mutation, since 58% melanomas with *TERT* promoter mutations also harbored the *BRAFV*600E mutation and *BRAF* mutation was present in 25% melanomas without *TERT* mutations.

Our results suggest that *TERT* promoter mutation plays a role in melanoma development. In cutaneous melanoma, *TERT* promoter mutations are associated with the presence of *BRAFV*600E mutations and poorer prognosis of the patients.

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Intronic *CDH1* variation landscape in HDGC syndrome – a place for causal alterations?

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Introduction: Hereditary diffuse gastric cancer (HDGC) syndrome, although rare, is severe, highly penetrant, difficult to diagnose and incurable. Forty-five percent of HDGC-families are associated with germline *CDH1*/E-cadherin coding alterations.[1-3] We recently implicated other gastric cancer (GC) susceptibility genes in ~5% of *CDH1*-negative families (submitted). Of the 50% that remain without molecular diagnosis, 2/3 present a phenotype of monoallelic CDH1 downregulation[4,5] and >90% display loss of E-cadherin protein expression in tumours.6 This led us to hypothesize that HDGC families lacking *CDH1*-coding mutations may harbour germline alterations in non-coding regions.

Methods and Results: The complete *CDH1* locus was screened in 90 *CDH1*-negative HDGC probands by NGS. In 35/90 probands, we found 42 true rare heterozygous germline non-coding variants (NCVs), 37 of which bioinformatically predicted to alter transcription factors binding sites (TFBSs). Of these, 12 NCVs could potentially impair the binding of known *CDH1* expression modulators. Moreover, 20/42 NCVs occurred in DNAseI-HSSs and 11/42 in areas highly conserved with the mouse genome. Importantly, *CDH1* germline allele-specific expression was assessed in 10 available single-NCV-carrying probands, five of which displayed massive mRNA monoallelic downregulation.

Conclusion: Our data suggest that at least some *CDH1* non-coding variants may cause germline *CDH1* monoallelic downregulation if they occur within regulatory sequences crucial for adequate E-cadherin expression control, namely at binding sites for *CDH1* expression modulators. Any novel pathogenic alteration identified in this cohort will warrant a complete redefinition of the screening methodology currently applied to HDGC families, and will provide important insights for the understanding of *CDH1* expression regulation.

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CANCER | POSTER 30

miR-128 increases DNA damage in acute myeloid leukemia cells

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MicroRNAs (miRs) are endogenous, small, non-coding RNAs that post-transcriptionally regulate gene expression by binding target mRNAs and typically inhibiting their translation. miRs have been shown to play an important role in cancer development and miR-128 has also been shown to be associated with cancer, namely leukemia. In particular, miR-128 was included in a miR-signature that allows discriminating between AML and ALL [1]. In addition, miR-128 was included in another miR-signature associated with failure to achieve complete remission in AML [2]. Nevertheless, all data that associates miR-128 expression with leukemia is derived from expression array analysis and no functional studies had been carried out. The aim of this work was to understand the function of miR-128 in AML cells and in their response to chemotherapy.

Cells from the HL-60 (AML) cell line were transiently transfected with control or miR-128 mimics. The miR-128 expression levels were quantified by real-time RT-qPCR. Cellular sensitization to doxorubicin or etoposide was assessed by Trypan blue exclusion assay. Cellular effect of miR-128 overexpression was further studied with the following assays: cell death (TUNEL and Annexin V/PI staining by flow cytometry); cellular proliferation (BrdU incorporation assay) and cell cycle profile (PI staining by flow cytometry). The expression levels of proteins involved in apoptosis (caspase-3, PARP), autophagy (Beclin-1, Vps34 and LC3) and DNA damage (γ-H2AX, 53BP1) were studied (Western Blot). DNA damage was also analysed with the Comet assay and by looking at γ-H2AX and 53BP1 foci formation (immunofluorescence microscopy).

Results showed that miR-128 overexpression caused a small decrease in viable cell number and an increase in TUNEL positive cells. This overexpression sensitized HL-60 cells to both doxorubicin and etoposide. Interestingly, it caused no alterations in cell cycle profile, cellular proliferation, apoptosis or autophagy-related proteins expression levels. Comet assay revealed an increase in the % of comet tail DNA in miR-128 transfected cells. This increase in DNA damage was confirmed by an increase in the DNA repair foci of γ-H2AX and 53BP1, together with an increase in the expression of both γ–H2AX and 53BP1 proteins.

Analysis of miR-128 expression in samples from PBMCs of 13 healthy donors and from bone marrow of 11 AML patients showed no statistically significant differences in the expression levels of this miR, although the levels in the AML samples were 1.87 times higher than in healthy donors.

In conclusion, overexpression of miR-128 increased sensitivity of HL-60 cells to etoposide or doxorubicin and increased TUNEL positive cells, possibly due to DNA damage induction. Regarding patient samples, a slight increase in the expression levels of miR-128 was found in AML bone marrow samples, when compared to PBMCs from healthy donors, suggesting that these patients may have an increased DNA damage. It would be interesting to analyze more patients and to correlate miR-128 levels in these patients with response to DNA damage-inducing agents.

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A lentivirus-based shRNA screen identifies integrator complex subunit 9 (INTS9) as a regulator of MCL1 alternative polyadenylation in human T cells

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Alternative polyadenylation (APA) originates multiple transcript isoforms due to the selection of different polyadenylation (pA) signals, increasing immensely the diversity of the transcriptome[1,2]. In recent years, APA have garner much interest as several studies have shown that abnormalities in the 3′-end processing mechanism are a common feature among many oncological, immunological, neurological and hematological diseases[3]. APA is also relevant under non-pathological conditions such as in the immune system. It was shown that upon T cell activation there is a global switch in the pA signal selection from a distal to a proximal pA signal, originating mRNAs with shorter 3'UTRs and, consequently, less prone to be regulated[4]. However, despite of its significant role in gene expression in human health and disease, the molecular mechanisms underlying the APA process remain surprisingly unclear. Here, we dissect the mechanisms and the molecular players involved in APA regulation by using T cells as a model. Using high throughput sequencing data we identified *MCL1* as a good candidate for studying APA in T cells, as its expression is altered upon T cell activation originating transcripts with shorter 3'UTRs. Moreover, *MCL1* is essential for embryogenesis and for the development and maintenance of T cells[5]. Using 3'RACE analyses, we confirmed that *MCL1* undergoes APA originating four mRNAs that differ only in the 3'UTR length. To identify the factors involved in *MCL1* APA, we used a lentivirus-based shRNA screen to knock-down (KD) polyadenylation factors, RNA-binding proteins and factors involved in transcription. The effect of the KD of each of these factors on *MCL1* pA signal selection was determined by qRT-PCR after lentiviral infection of a human T cell line (Jurkat E6.1). Our initial screening identified *INTS9* as a regulator of *MCL1* APA, given that its KD leads to the inhibition of *MCL1* distal pA signals. INTS9 has been shown to be involved in small nuclear RNAs 3'end processing and to directly interact with the C-terminal domain (CTD) of RNA polymerase II[6]. However, no previous role for *INTS9* was identified in mRNA APA. Taken together **our results show that INTS9, which associates with RNA** polymerase II, play an important role in APA regulation and open up new avenues in understanding this mechanism.

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CANCER | POSTER 32

Adipocyte secretome influence on glioma cells

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Aims: Glioma is the most frequent form of malignant brain tumor in the adults and childhood. There is a global propensity toward a higher incidence of gliomas in well-developed and industrialized countries. Simultaneously, obesity is reaching epidemic proportions in such developed countries. It has been widely accepted that obesity may play an important role in the biology of several types of cancer. We have developed an *in vitro* method for the understanding of obesity influence on glioma mouse cells (Gl261).

Methods: 3T3-L1 mouse pre-adipocytes were induced to the maturity. Conditioned medium was harvested and applied on Gl261 cultures. Using two-dimension electrophoresis, it was analyzed the proteome content of Gl261 in the presence of conditioned medium (CGl), as well as in its absence (NCGl). The differently expressed spots were collected and analyzed by means of mass spectroscopy (MALDI-TOF-MS).

Results: Statistically significant expression pattern changes were observed in eleven proteins and enzymes. RFC1, KIF5C, ANXA2, N-RAP, RACK1 and citrate synthase were overexpressed or uniquely present in the CGl. Contrariwise, STI1, hnRNPs and phosphoglycerate kinase 1 were significantly underexpressed in CGl. Aldose reductase and carbonic anhydrase were expressed only in NCGl.

Conclusions: Our results reveal that obesity remodels physiological and metabolic behavior of glioma cancer cells. Moreover, proteins found differently expressed are implicated in several signaling pathways related to matrix remodeling control, proliferation, progression, migration and invasion. In general, our results support the idea that obesity may increase glioma malignancy, even though some interesting paradox finding were also reported and discussed.

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Extracellular vesicles - shuttles of epithelial to mesenchymal to epithelial transitions

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Introduction: Epithelial to mesenchymal and mesenchymal to epithelial transitions (EMT/MET) can promote cancer cell plasticity and fuel tumour initiation, metastatic spread and colonization.
Objective: We aimed at understanding whether EMT/MET cells use extracellular vesicles (EVs) to perpetuate aggressive phenotypes that ultimately cause metastasis.
Methods: A TGFβ1-induced EMT/MET model was generated using MCF10A cells and characterized by qRT-PCR/immunofluorescence for the presence of epithelial (E-cadherin, Occludin) and mesenchymal (Vimentin, Fibronectin, Snail, Twist) markers. EVs were isolated from the conditioned medium of epithelial (E), mesenchymal (M) and reversed-epithelial (RE) cells by differential ultracentrifugation and characterized by TEM and WB. Angiogenic activity of E, M, RE cells and corresponding EVs was assessed by CAM *in vivo* assay.
Results: M cells displayed loss of epithelial markers and concomitant gain of mesenchymal markers in comparison with E cells. RE cells partially recovered the epithelial features observed in E cells. EVs isolated from E, M and RE cells had a diameter size of 30-150 nm and expressed CD9, CD81 and Tsg101. An increased angiogenic potential was observed in M cells compared to E and RE cells. In addition, M cells-derived EVs recruited more vessels than E and RE cells-derived EVs.
Conclusion: We established a dynamic *in vitro* model that recreates the EMT and MET programs and found that EVs were able to recapitulate the angiogenic behaviour of the corresponding E, M and RE cells.

Understanding the function of *sox21b* in zebrafish pancreas development

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The vertebrate endocrine pancreas has several important roles, among them controlling blood glucose levels. Pancreas malformation or malfunction usually results in important human diseases such as diabetes, pancreatitis or pancreatic cancer. In order to find new therapeutic solutions for these diseases, it is imperative to understand the genetic networks that determine pancreas organogenesis and function. With this work we aim to study the function of new genes involved in vertebrate pancreas development. Using the zebrafish as a model system we have performed a tissue specific *in vivo* enhancer trap screen by mobilizing a Tol2 based transposon in the zebrafish genome, named the Expression Disruption vector (EDscreen)[1]. Using this approach we have screened for ED lines that showed expression in the pancreas. One of the identified lines was ED301. By performing inverse PCR we have mapped the ED301 insertion, which revealed to be 50 kb upstream of the gene *sox21b*. When comparing the expression pattern of *sox21b* with the expression of the ED301 reporter genes we detected a similarity in these patterns, suggesting that the ED301 enhancer trap line is detecting the transcriptional regulatory landscape of *sox21b*. This gene belongs to the Sox family, which encodes for crucial transcription factors for embryonic development[2]. Dysregulation of Sox factors have been associated with some human diseases[3]. To analyze *sox21b* function in pancreas development and to determine its epistatic position in these developmental networks we are using two loss of function methods: morpholinos[4] and site-directed mutagenesis (Cripr-Cas9)[5]. These functional assays revealed a decrease in the expression area of insulin, suggesting the requirement of *sox21b* for proper β-cell differentiation. So far preliminary results suggest the finding of a new gene with an important role in pancreas development.

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CANCER | POSTER 35

FoxM1 repression during human chronological aging compromises mitotic fidelity

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So far, the largest single risk factor for developing cancer is advancing age [1], and interestingly aging has been associated with aneuploidy [2]. It is paradoxal how can aging, characterized by reduced cell proliferation, promote late-life cancer, characterized by uncontrolled cell proliferation. Studies have identified genes whose expression levels change with age-related phenotypes and diseases [3,4]. Among these are several mitotic genes. We began by examining the thus far elusive effect of chronological aging in mitotic fidelity. Using both long-term phase-contrast and high-resolution spinning–disk confocal live cell imaging, we followed cell cycle behaviour of individual young, middle and old age human dermal fibroblasts from Caucasian males. We found old cells to delay in prometaphase. This delay is dependent on the activation of the spindle assembly checkpoint consistently to the presence of misaligned chromosomes and spindle defects observed. Also, aneuploidy was found to increase with advancing age. Concurrently to the pleiotropic mitotic phenotype of human aged cells, we detected decreased levels of the transcription factor FoxM1, which primarily drives the expression of G2/M specific genes [5]. Following expression of a constitutively active form of FoxM1, the levels of several mitotic proteins were restored in human elderly fibroblasts, and the age-associated mitotic phenotypes significantly reverted. In sum, we show that FoxM1 transcription factor is downregulated during human chronological aging and that its repression accounts for the mitotic and karyotypic defects found in human aged cells. Our findings uncover a new molecular mechanism that could be explored to both extend longevity and prevent aging diseases such as cancer.

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P-cadherin represses cell-cell adhesion mediated by E-cadherin in breast cancer cells: an AFM analysis

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E- and P-cadherins are cell-cell adhesion molecules crucial for the maintenance of normal epithelial tissue architecture. In epithelial cancers, E-cadherin acts as an invasion suppressor protein, being lost in highly infiltrative tumours. In contrast, P-cadherin is overexpressed in a significant fraction of carcinomas endogenously expressing E-cadherin, inducing collective cell invasion and acting as a repressor of the normal adhesion complex. Recently, we found that P-cadherin-induced invasion is dependent on Src-activity, which regulates the expression and trafficking of E-cadherin. Based on this data, our goals were: 1) to characterize the morphological and mechanical properties induced by P-cadherin overexpression in E-cadherin-positive cancer cells; and 2) to evaluate these properties in P-cadherin-overexpressing cells upon Dasatinib treatment, an inhibitor of Src activity.

Materials and Methods: Two E-cadherin-positive breast cancer models have been used: MCF-7/AZ cell line, retrovirally transduced to encode P-cadherin cDNA (MCF.7/AZ.Mock and MCF-7/AZ.P-cad); and BT-20 cell line, endogenously expressing high levels of P-cadherin. BT20 were transfected with small interfering RNA (siRNA) against P-cadherin. Dasatininb treatment was applied with a final concentration of 100nM. Atomic force microscopy (AFM) was performed in both cell models, with and without Dasatinib treatment. Cells were scanned and AFM images were analysed, yielding maximum height, area and volume values for the different cell. Differences on cell stiffness and cell-cell adhesion forces were evaluated by AFM-based force spectroscopy.

Results and Discussion: By AFM, we found that MCF-7/AZ.Pcad cells present significantly higher area and volume, as well as a decrease height. Accordingly, BT20 transfection with P-cadherin siRNA lead to a significant reduction of the cell's area and volume, and an increased height. MCF-7/AZ.Pcad cells also presented a lower Young's Modulus, which indicates higher cell elasticity, whereas P-cadherin silencing in BT-20 induced a significant increase in the Young's Modulus value, revealing a decreased elasticity. Interestingly, the treatment of P-cadherin-overexpressing cells with Dasatinib induced the same results in both models: a reduction in the cell's area and volume, as well as an increase in the cellular height and Young's Modulus (less elastic cells). Concerning cell-cell adhesion, we found that the work necessary to separate MCF-7/AZ.P-cad cells was significantly lower than for MCF-7/AZ.Mock cells. Accordingly, P-cadherin silencing in BT-20 cells induced increased values of work relative to the respective control. The same trend was obtained after Dasatinib treatment in both P-cadherin-overexpressing cell models.

Conclusions: AFM measurements demonstrated that P-cadherin represses the normal cell-cell adhesion mediated by E-cadherin in cancer cells, justifying the increased invasive phenotype besides the cell-cell adhesion maintenance. These results are supported by the morphological characteristics adopted by P-cadherin-overexpressing cells, which present an increased cell volume and elasticity, parameters usually found in invasive cancer cells. The treatment with Dasatinib reverts the P-cadherin-induced phenotype, allowing cancer cells to adopt a more "epithelial-like" behaviour and being less invasive.

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CANCER | POSTER 37

Oseltamivir phosphate increases canine mammary cancer aggressiveness

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Oseltamivir phosphate is a widely used anti-influenza sialidase inhibitor. Sialylation, governed by sialyltransferases and sialidases, is strongly implicated in the oncogenesis and progression of breast cancer. In this study we evaluated the biological behavior of canine mammary cell tumors upon oseltamivir phosphate treatment (a sialidase inhibitor) *in vitro* and *in vivo*. Our *in vitro* results showed that oseltamivir phosphate impairs sialidase activity in a dose-dependent manner leading to increased sialylation in CMA07 and CMT-U27 canine mammary cancer cells. Surprisingly, oseltamivir phosphate stimulated, in a dose-dependent manner, CMT-U27 cell migration and invasion capacity *in vitro*. CMT-U27 xenograft tumors of oseltamivir phosphate-treated nude mice showed increased sialylation, namely α2,6 terminal structures and SLe(x) expression. Remarkably, a trend towards increased lung metastases was observed in oseltamivir phosphate-treated nude mice. Taken together, our findings revealed that oseltamivir impairs canine mammary cancer cell sialidase activity, altering the sialylation pattern of canine mammary tumors, and leading, surprisingly, to *in vitro* and *in vivo* increased mammary tumor aggressiveness.

CANCER | POSTER 38

Dissecting the role of Polo kinase in the regulation of kinetochore-microtubule interactions

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Cell division supports organism viability and has to be precisely coordinated to ensure the correct transmission of the genomic content. Before chromosome segregation, spindle microtubules interact with kinetochores, a process that is monitored by the spindle assembly checkpoint (SAC), which acts as a surveillance mechanism that prevents mitotic exit before all kinetochores become bi-oriented. Polo kinase has been implicated in both processes, either by promoting dynamic interactions between chromosomes and microtubules or by contributing to SAC regulation. However, a consensual mechanism of action remains to be described. Here, we searched for Polo targets by screening through a list of candidate genes that, when down regulated, could rescue the dominant phenotype caused by expression of the constitutively active PoloT182D mutant resulting after driving expression in a tissue-specific manner. We found as potential substrates proteins classically involved in SAC response, but further dissection of PoloT182D mitotic outcome revealed a possible pathway by which Polo may regulate kinetochore microtubule interactions. These observations can help to define the role of Polo kinase in supporting faithful chromosome alignment and segregation.

Establishment of a cellularized artificial model of the gastric wall

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Cellular permeation models are tools of the upmost importance when studying the application of new drugs for therapeutic use, since they help predict the physiological effects, as well as the drug absorption rate and metabolism of new molecules, making them an easy, reproducible, ethical and cost-effective method for assessing drug-absorption and toxicity mechanisms [1]. Although some *in vitro* permeation models were already developed for the intestinal, pulmonary, nasal, vaginal, rectal, ocular and skin tissues, including triple co-culture *in vitro* models of the intestine [2-4], surprisingly few established *in vitro* permeation models of the gastric wall exist, especially due to the difficulties in maintaining primary gastric cultures [5], and among these only simple co-cultures were used. Therefore, the development of a triple co-culture model of permeation for the stomach is of paramount importance for the evaluation of new therapeutic agents.

The main objective of this project was to optimize and establish a triple co-culture *in vitro* cellular model of the gastric wall to replicate its functional and morphological architecture with application in permeability, toxicity and functional assays. In order to accomplish this, a triple co-culture model of the stomach was established, including fibroblasts, macrophages and epithelial cells. The integrity of the membrane formed was assessed over time, the permeability of the created barrier model to the passage of substances was quantified and the model was further morphologically and structurally characterized.

NST20 fibroblasts were cultured upon transwell membranes, which were then either coated with Matrigel™ or PuraMatrix™, and MKN28 epithelial cells were seeded on top of this coating, to mimic both the mucosa's epithelium and lamina propria. Permeability assays using FITC-dextran were used to assess the model's integrity. The optimal cell densities to build the model were determined, namely of 5×10^3 NST20 fibroblasts, 5×10^3 THP-1 derived macrophages and 5×10^4 MKN28 epithelial cells, when cultured in either Matrigel™ or PuraMatrix™. The optimized barrier model yielded moderately high trans-epithelial electric resistance values of about 200 $\Omega.cm^2$, which correlate to a high membrane integrity, and low apparent permeability to FITC-dextran (approximately 1×10^{-6}cm/s), as desired. The model was further characterized structurally by fluorescence, confocal and transmission electron microscopy.

The model herein developed constitutes a step forward in the development of *in vitro* model systems of the stomach, by exhibiting rudimentary compositional and functional characteristics of the gastric wall. Considering the present results, the application of the developed model as a cellular *in vitro* permeation model for the gastric wall seems viable, although further optimization and functional and structural characterization is required.

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CANCER | POSTER 40

Hox Genes and the Evolution of Vertebrate Limbs

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According to the fossil record, tetrapods were the first vertebrate group with the ability to walk on land. Adaptation to land was enabled by the autopod, a multi-finger extremity that evolved from sarcopterygian fish fins, by sequential expansion and elaboration of their appendicular skeleton. In the tetrapod ancestors, addition of novel distal endoskeleton elements co-occurred with the reduction of the ectodermal fold present in the distal end of fish fins. Modulation of 5´HoxD gene transcription has long been suggested as a possible evolutionary mechanism involved in these morphological transformations. Recently, we have proven that gain of *hoxd13a* function during zebrafish fin development was shown to cause increased proliferation and distal expansion of chondrogenic tissue, accompanied by finfold reduction (1). Here we will explore how hoxd13a transcriptional modulation affects the expression of its downstream targets. For our analyses, we selected genes described to be strongly involved in limb development and shown to bind to Hoxd13 in Chip-to-Chip assays. The role that these genes might play in the overall picture of the fin-to-limb transition will be discussed. In the future we also intend to explore how Hoxd13 modulation impacts the fin transcriptome in order to obtain a wider view of the genetic players that participate in appendage development, and that might have been important for the fin-to-limb transition, while explaining also congenital limb malformations.

Detection of somatic alterations in plasma from lung cancer patients

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Tumor-specific (somatic) mutations in plasma can serve as biomarkers for tumor detection, monitor tumor response to specific therapies, detect residual disease after surgery, and long-term follow-up. The intrinsic low abundance of circulating cell-free tumor DNA (cfDNA) makes the detection and quantification of such mutations in plasma a challenging task. This small scale study aims to establish a comprehensive strategy to be used in the detection of clinical relevant somatic alterations in plasma of lung cancer patients.

Plasma samples obtained at different stages of disease progression and/or treatment were collected from a group of 11 lung cancer patients and used to isolate cfDNA. Genetic alterations in the EGFR gene (p.E746_A750del and p.L858R) identified at diagnosis in tumor biopsies were used as surrogate markers to optimize and validate the next generation sequencing strategy and data analysis workflow. The Ion AmpliSeq Colon and Lung cancer panel was used to analyze hotspot and targeted regions of 22 genes implicated in colon and lung cancers, including the EGFR gene mutations mentioned. The amplified products were used to prepare libraries and were sequenced using the Ion PGM system. Quantitative real time PCR and digital PCR assays were used to confirm selected results.

Tumor derived genetic alterations could be identified in as little as 10ng of cfDNA. EGFR alterations identified in cfDNA mirrored the alterations identified in all tumor biopsies. EGFR alterations with allelic frequencies as low as 3% could be detected in cfDNA. Additionally, in case of samples collected after therapy, a clear decrease in the cfDNA allelic frequency of the EGFR mutation, that made the patient eligible for therapy, was observed. Furthermore, the screening of a larger panel of genes allowed the identification in two cases of additional gene mutations (e.g. MET and KRAS) that may have impact in the clinical management of patients.

In this study, we demonstrate the capacity to identify clinically relevant somatic EGFR mutations in plasma. The possibility to assess information from a larger panel of genes makes this strategy attractive for further optimization of treatment options. The strategy is now being extended to a larger cohort of patients to push forward the concept of liquid biopsy in the clinical management of cancer patients.

HSP90 Cleavage associates with oxidized proteins accumulation upon proteasome inhinition

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Oxidative stress, OS, has been associated to a variety of phenomena as cancer progression, neurodegeneration and ageing itself. At a molecular level, OS leads to protein carbonylation, a non-enzymatic irreversible event and common feature of aged cells. Carbonylated proteins are dysfunctional and can accumulate, in the form of protein aggregates that alter cellular functionality [1].

To cope with carbonylated proteins, cells employ the proteasome, the main non-lysosomal structure for carbonylated proteins turnover. However, if the degrading rate is inferior to carbonylated proteins formation rate, protein aggregates form [2].

In a previous study, in oxidative stress challenged Jurkat cells we could verify that cytoplasmatic actin becomes heavily carbonylated and forms oxidized actin aggregates, which lead to proliferation impairment and proteasome activity diminishment, similar to senescence like states [3]. Because under these oxidative conditions there is a proteostasis disturbance, such as oxidized proteins (especially actin) accumulation and proteasome activity impairment, Hsp90 involvement was studied. Hsp90, a molecular chaperone, assists oxidized proteins degradation and also protects the 20S proteasome from oxidative inactivation [4, 5]. We reasoned that the mechanism by which protein aggregates can form, mainly happens due to Hsp90 lesser functionality, which we attribute to cleavage. In our study, we were able to verify that cleaved Hsp90 is present in protein aggregates and that it occurs before actin insolubilization, verified after fractioning soluble and insoluble cellular extracts.

We are convinced, supported by our data, that cleaved Hsp90 is an important intervenient for oxidized protein accumulation and proteasome inactivation.

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CANCER | POSTER 43

Glycoprofiling of serous ovarian tumours is a promising strategy for developing new diagnostic tools

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Introduction: Aberrant O-glycosylation of mucins is consistently associated with human carcinomas, including ovarian carcinomas. Alterations in the expression of simple mucin-type antigens (Tn, sialyl-Tn and T) are typical changes of O-glycosylation in cancer. Little is known about the carriers of these antigens in human carcinomas, with evidence indicating mucin 1 (MUC1) and mucin 16 (MUC16) as putative "transporters". Our hypothesis is that, depending on the carrier protein, Tn, sTn and T may have differential biological significance and also higher cancer-specificity.

Materials and methods: We evaluated a series of benign (n=27), borderline (n=14) and malignant (n=21) serous ovarian tumours, using whole sections or TMAs. To access the glycopeptide combinations between simple mucin-type carbohydrates and MUC1 and MUC16 mucins, a brightfield Proximity Ligation Assay (PLA) was used. We evaluated the expression of different PLA pairs or combinations of PLA pairs according to histological diagnosis and computed the sensitivity (Sn), specificity (Sp) and positive and negative predictive values (PPV and NPV-, respectively), for distinguishing between malignant/borderline serous tumours and benign conditions.

Results and discussion: PLA pairs MUC16/STn, MUC1/STn and MUC16/Tn were not detected in benign lesions and were more frequently observed in malignant (71%, 62% and 48%, respectively) than in borderline lesions (36%, 29% and 21%, respectively) . Expression of PLA pair MUC1/Tn occurred only among malignant cases (62%). MUC16/T was expressed more frequently among malignant and borderline than in benign lesions (33%, 36% and 7%, respectively), and MUC1/T among malignant lesions (38%, 7% and 4%, respectively). Considering the positivity for combinations of PLA pairs that maximize sensitivity and specificity, the positivity to MUC16/STn or MUC1/STn, the Sn, Sp, PPV and NPV were 69%, 100%, 100% and 71%, respectively. A test based on the positivity for MUC16/STn or MUC1/STn or MUC16/Tn or MUC1/Tn, further increased Sn to 74% and NPV to 75%.

Conclusions: Our results show that glycopeptide identification of MUC1 and MUC16 together with the Tn and STn glycan structures is promising for distinguishing between malignant/borderline serous ovarian tumours from benign lesions. Further research is warranted towards the development of serum assays based on this glycoprofile signature.

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ER stress response activation during cellular senescence

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The aging process is characterized by a progressive accumulation of damaged biomolecules in the endoplasmic reticulum (ER), as a result of the increased oxidative stress that accompanies cellular senescence. Being so, ER homeostasis and function are likely to be affected during senescence. Moreover, it is known that in stressful conditions, cells activate a protective ER stress response, mediated by ER transmembrane proteins (IRE1, ATF6 and PERK), in an attempt to restore proteostasis and maintain cell function. The occurrence of such events in human cellular models of senescence had not been addressed before. So, we hypothesized that in replicative senescence (RS) and hydrogen peroxide (H_2O_2) or copper sulfate ($CuSO_4$) stress-induced premature senescence (SIPS) cellular models the ER chaperoning mechanisms would be impaired and ER stress response would be activated.

Protein and mRNA levels of key ER chaperones/enzymes as well as IRE1-, ATF6- and PERK-mediated ER stress response activation are shown here on WI38 human fibroblasts senescent models. In $CuSO_4$-SIPS and RS conditions, BiP, calnexin, PDI and Ero1 levels are adjusted to restore proteostasis and IRE1-, ATF6- and PERK-mediated ER stress responses are activated. However, H_2O_2-SIPS do not exhibit IRE1 and ATF6 pathways activation, but a PERK-mediated upregulation of CHOP, showing that $CuSO_4$-SIPS mimics better the molecular events associated to proteostasis and ER response of RS than H_2O_2-SIPS. Moreover, it is shown here that ER stress activation is required for the induction of senescence in both SIPS models, since PERK and IRE1 specific inhibitors led to a decrease in senescence-associated beta-galactosidase appearance. In CuSO4-SIPS, the decrease in senescence levels is associated with PERK-driven, but IRE1 independent, cell cycle arrest while H_2O_2-SIPS cell proliferation inhibition is PERK independent.

In conclusion, these results add a step further on the molecular mechanisms that regulate senescence induction; moreover, they validate $CuSO_4$-SIPS model as a useful tool to study cellular stress responses during aging, hoping to postpone age-related health decline.

Effect of TUDCA in an obesity model of brain tumor cells with Radiotherapy

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Introduction: The bile acids, such as acid tauroursodeoxycholic, have been demonstrated to have neuroprotective effects in both several neurodegenerative diseases and ischemia models. The brain tumors cells have a specific microenvironment, developing both oxidative stress and inflammation. Previous studies, in brain tumor, indicated that obesity may be related with a decreased resistance to radiation and increased redox status. Since Radiotherapy is the most commonly treatment used in this type of tumor, we propose to explore the influence of the obesity in radiated glioma cells in the presence with TUDCA.

Methods: BC3H1 glioma cells were treated with TUDCA (25μM) in serum-free DMEM or conditioned media (CM) from differentiated 3T3-L1 adipocytes. Afterwards the cells were irradiated with a total dose of 2 Gy. Subsequently BC3H1 viability was evaluated, by MTT assay; the oxidative stress by TBARS and the total antioxidant status (TAS), after 4 and 12 hours.

Results: We observed an increase in viability in all cells treated solely with 3T3-L1 CM. Interestingly, we observed an increase of the total antioxidant status in the irradiated cells compared with the non irradiated cells, at the same time there is an increase of oxidative stress in irradiated cells and with TUDCA 4 hours after radiotherapy. Nevertheless, 12 hours after radiotherapy, the oxidative stress decreased with TUDCA and total antioxidant status increase in the cells with CM, TUDCA and radiation.

Discussion / Conclusion: The 3T3-L1 MC increases the cell viability in the presence of radiation or not, after 12 hours expose. The oxidative stress and the total antioxidant status don't play an inverse correlation in this study, probably because TUDCA and CM have a direct influence in the radiation action. At this point, we need to deepen the studies to understand the TUDCA's radiossensitazing mechanism of action.

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Role of osteopontin isoforms in thyroid cancer

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Background: Thyroid cancer (TC) is the most common malignancy of the endocrine system (1). Osteopontin (OPN) is one of the gene products aberrantly expressed in TC (2, 3), but the contribution of each OPN three splicing isoform (OPNsi), named as OPNa, OPNb and OPNc, is currently unknown.

Objectives: To investigate the expression profile of OPNsi in TC tissue samples and to correlate its expression with molecular and clinicopathologic features.

Methods: In order to address the putative roles of OPNsi in TC, we overexpressed OPN isoforms in TC derived cell lines. The expression profiles of total OPN (OPNt) and OPNsi in eight TC cell lines (TPC1, KAT4, Hth74, XTC1, 8505c, K1, BCPAP and c643) and in thyroid tissue samples were evaluated by q-RT-PCR. c643 and 8505c cells were transfected with vectors containing OPNsi (A, B, C) as well as empty vector, and stable overexpressing clones were selected with geneticin treatment. Cell proliferation was evaluated by BrDU assay and cell migration was evaluated by wound-healing assay.

Results and conclusions: We found that the OPNa and the OPNb isoform transcripts are expressed in higher levels in papillary thyroid carcinoma (PTC) samples than in non-tumoral thyroid, adenomas and follicular thyroid carcinoma tissues. Conversely, OPNc isoform transcript levels are similar among samples from the aforementioned pathologies. In PTC tissues, OPNa and OPNb expression levels were higher in tumours harboring $BRAF^{V600E}$ mutation than in wild-type BRAF tumor samples ($p = 0.006$ and $p = 0.039$, respectively). Tumor size was significantly correlated with OPNt expression levels ($p = 0.023$) and absence of encapsulation was significantly associated with higher levels of OPNa expression ($p = 0.042$). In eight distinct TC cell lines, we observed differential expression of the three OPNsi, of which cell line c643 expressed the lower levels of OPNsi. Higher proliferation and migration rates were associated with c643 and 8505c cells overexpressing OPNa, when compared to empty vector control clones. Taken together, our data indicate that a) both OPNa and OPNb are overexpressed in PTC samples, b) OPNa is variant that is significantly associated to promotion of cell growth and migration advantages in c643 and 8505c TC cells.

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Functional analysis of two new protein homologues that interact with CLASPs during mitosis.

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Chromosome movement during mitosis is a phenomenon mediated by a cellular machine called mitotic spindle, a bipolar structure built by microtubules (MTs). MT dynamics is regulated by MT-associated proteins (MAPs), such as MT plus-end tracking proteins (+TIPs). CLIP-associated proteins (CLASPs) belong to this family and are highly conserved among eukaryotes. These proteins interact with MTs regulating mitotic spindle bipolarity, chromosome segregation.
Recently, a proteomic survey for CLASP1 interactors during mitosis brought to our attention two proteins, which have not been characterized. So far, we named them as CLK1 and CLK2 (standing for CLASPs-like KIAA). They consist of two high molecular weight proteins that localize to microtubules during interphase, the mitotic spindle and midbody during mitosis.
Regarding the role of these proteins in mitosis, we found out that CLK1 knockdown leads to a prolonged metaphase arrest. Conversely, CLK2 depletion causes a shorter mitotic delay, displaying chromosome missegregation and lagging chromosomes. Interestingly, simultaneous depletion of both CLKs results in a mixed phenotype, indicating that both CLKs display distinct functions in mitosis. Additionally, CLASPs depletion seems to downregulate CLK1 expression, both in interphase and mitosis.
CLKs are new proteins family which role in mitosis has never been studied before. So far, our study has shown that these two proteins are important players in maintenance of the mitotic fidelity.

The role of cancer stem cells in breast cancer metastasis

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Introduction: Breast cancer is the most common malignant disease in western women (1), being metastases the most important cause of death (2). Recent studies have suggested that breast cancer stem cells (BCSCs), a subset of cancer cells with stem cell characteristics, mediate tumorigenic potential as well as distant metastases (3), being resistant to radiation and chemotherapy, and thus contributing to both the heterogeneity of breast cancers and the relapse of tumors after treatment (4). Although several BCSC markers have been already described, it is still unclear whether these proteins are enriched in metastatic lesions compared to primary tumors, as well as if these can be used as relevant targets in untreatable metastatic breast cancer (5).

Aim: Thus, the aim of this work was to investigate the expression of BCSC markers in breast cancer metastasis, in order to search for their potential use as clinical targets in advanced breast cancer.

Materials and Methods: The expression of some BCSC markers was assessed by immunohistochemistry in clinical samples of primary breast carcinomas and matched lymph node metastases, as well as in unmatched brain metastasis. The results were correlated with the clinicopathological data and patient outcomes. BCSC markers were also evaluated in two human brain tropic cancer cell lines obtained from P. Steeg´s laboratory - NIH (National Institute of Health, Bethesda, USA). Mammosphere-forming ability generated from brain tropic and parental cell lines were compared. The expression levels of BCSC markers were evaluated by western blot, RT-PCR and flow cytometry.

Results and Discussion: Our results showed that the expression of P-cadherin in metastatic lymph nodes was significantly enriched and associated with worse DFS and OS. Furthermore, P-cadherin expression was associated with the expression of the CD44 and CD49f BCSC markers in metastatic lymph nodes. However, no statically significant association was found between the expression of CD49f and CD44 and patient outcome. Additionally, P-cadherin, CD44 and CD49f were highly expressed in brain metastasis.

Using the metastatic breast cancer cell model, we verified an enrichment of the CD24 marker in brain tropic cells, potentiating the epithelial-like phenotype of CD44+CD24+ compared to the parental cell line. Additionally, brain tropic breast cancer cells exhibited a higher mammosphere forming efficiency than the parental cell line, as well as an altered profile of the cancer stem cells/metastatic markers EpCAM, CXCR4, CD49f. We still found that Src was hyperactivated in brain tropic cells and significantly expressed in human brain metastases. Mammosphere assay was still performed to explore the effect of the Src inhibitor dasatinib on stem cell activity. Dasatinib markedly reduced the number of mammospheres of brain tropic cell lines, with no effect in the parental cell line. Interestingly, dasatinib treatment also induced a higher rate of cell death in brain tropic cells, contrarily to the parental cell line, which showed substantially more resistance to the dasatinib effect.

Conclusions: This preliminary data suggests that BCSC signature may serve as a potential biomarker for breast cancer progression and a target for treatment.

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CANCER | POSTER 49

RNA-binding proteins regulate *polo* poly(A) usage through binding to a conserved USE

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Alternative polyadenylation (APA) has a critical role in pre-mRNA processing and important implications for gene expression regulation. Using a *Drosophila* mutant we have previously shown that RNA polymerase II elongation rate affects polyadenylation site (pAs) selection in several genes. One of these is the cell cycle regulator *polo*, with two pAs in the 3'UTR (pA1 and pA2) producing mRNAs that differ in their 3' UTR length and yield distinct protein levels. We have also shown that these polo APA events have functional consequences on cell proliferation and organism development.

We have now identified some of the molecular players involved in polo mRNA 3' end formation. Using transgenic flies, we identified a conserved upstream sequence element (USE) localized upstream of *polo* pA1, necessary for mRNA 3' end formation *in vivo*. By UV crosslinking assays we mapped HuR/Elav and PTB/Hephaestus binding sites in *polo* USE and show that depletion of Elav and Heph in *Drosophila* S2 cells results in an increase in pA1 site usage. In mammals, USEs act as regulatory elements involved in pre-mRNA cleavage and polyadenylation, but these elements were yet to be identified in *Drosophila*. Taken together our *in vivo* and *in vitro* data reveals that HuR/Elav and PTB/Hephaestus regulate *polo* APA through binding to a conserved USE.

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CENP-E and tubulin detyrosination drive congression of peripheral polar chromosomes

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Accurate chromosome segregation during cell division in metazoans relies on proper chromosome congression at the equator. Chromosome congression is supported by the coordinated action of motor proteins that slide misaligned chromosomes along pre-existing spindle microtubules. These proteins include the minus end-directed kinetochore motor Dynein, and the plus end-directed motors CENP-E at kinetochores and chromokinesins on chromosome arms. However, how these opposite and spatially distinct activities are coordinated to drive chromosome congression remains unknown. Here we used RNAi, chemical inhibitions, kinetochore tracking and laser microsurgery to uncover the functional hierarchy between kinetochore and arm-associated motors, exclusively required for congression of peripheral polar chromosomes in human cells. We show that Dynein poleward force counteracts chromokinesins to prevent stabilization of immature/incorrect end-on kinetochore-microtubule attachments and random ejection of polar chromosomes. At the poles, CENP-E becomes dominant over Dynein and chromokinesins to bias chromosome ejection towards the equator. Finally, we show that experimental inhibition of tubulin detyrosination in living human cells dissociates CENP-E from microtubules and impairs congression of peripheral polar chromosomes, similarly to CENP-E depletion/inhibition. This bias for detyrosinated microtubules explains the dominant and selective role of CENP-E in sliding polar chromosomes exclusively towards the equator.

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Decellularized matrices derived from human colorectal cancer fragments modulate macrophage differentiation

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Tumour complexity, either referring to other cells types, soluble factors and extracellular matrix (ECM) components, is a topic that has been widely studied in the last decade. The fact that a cancer is much more than just tumour cells is being perceived as a great opportunity for cancer prevention and treatment. In this context, macrophages emerged as modulators of cancer progression, regulating breast cancer cell migration, invasion and metastasis. These are highly plastic immune cells, being able to adopt a myriad of polarization phenotypes, depending on the molecular context. In a rather simplistic vision, macrophages have been described as key elements for carcinogenesis, preventing the establishment and spreading of cancer cells – M1 macrophages – or supporting tumour growth and progression – M2 macrophages. We are particularly interested to elucidate how ECM components modulate macrophage polarization.

Therefore, we developed an innovative 3D-organotypic model, by decellularizing human colorectal cancer (CRC) tissue fragments and repopulating them with monocytes, mimicking more closely the natural tumour microenvironment. DNA quantification and DAPI staining confirmed the efficiency of decellularization. SEM analysis allowed visualization of the ECM fiber meshwork while Hematoxylin-Eosin staining revealed that decellularized fragments retained the original tissue histological features. Decellularization reduced significantly the glycosaminoglycans content without affecting other ECM components such as collagen, laminin or fibronectin. Repopulation experiments, using primary human monocytes, clearly evidenced that monocytes are able to colonize these decellularized matrices and to differentiate into macrophages within the fiber network. Most interestingly, normal and tumour-derived matrices seem to retain, at least partially, their endogenous biological properties by distinctly modulating macrophage differentiation. Our results consistently reveal that macrophages repopulating normal decellularized matrices secrete higher levels of IL-6 and present higher MMP-9 activity, while expressing more CD163, than macrophages repopulating tumour-derived decellularized matrices.

Elucidating the role of tumour cells and of ECM components, derived from the tumour microecosystem, on macrophage differentiation and polarization, opens new perspectives to the design of novel therapeutic strategies targeting macrophages.

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Nol12 repression leads to nucleolar stress, proliferation arrest and p53 activation

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The ability of cells to grow, proliferate and differentiate is influenced, at its basis, by ribosome biogenesis and activity. Besides its role as the ribosome factory, the nucleolus is now also considered to be a multifunctional regulatory compartment involved in RNA processing events, sensing of cell stress, and cell cycle and apoptosis regulation. When stimulation of ribosome biogenesis is blocked or uncoordinated, cells undergo nucleolar/ribotoxic stress. In mammalian cells, accumulated or relocated ribosomal proteins, such as RPL5 and RPL11, cause p53 stabilization by binding and inhibiting MDM2, an E3 ubiquitin ligase that inhibits p53 activity through proteasome-mediated degradation. Several recent papers have addressed the role of p53 activation in the cellular damage, cell-cycle arrest, and apoptosis caused by distinct nucleolar stress conditions. However, the contribution of p53-independent pathways has been reported, although it remains poorly understood. In the *Drosophila* model, our previous work showed that knocking down Viriato/Nol12 (a 5´-3´RNA exonuclease involved in rRNA processing) causes nucleolar stress, cell growth deficit and apoptosis, in a p53-independent manner [1]. Interestingly, our preliminary results show that Nol12 depletion in human fibroblasts results in a decrease of proliferation rate and upregulation of p53 expression, distinctly to the *Drosophila* model [2]. Therefore, these results point Nol12 as an important player for both nucleolar regulation and cell proliferation. Additionally, they emphasize the importance to study the function of Viriato/Nol12 in both *Drosophila* and human models in order to highlight the sensor and effector mechanisms involved in both p53-dependent and p53-independent nucleolar stress pathways.

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CANCER | POSTER 53

Organelle-excluding compartmentalization during spindle assembly confines key mitotic regulators and promotes mitotic fidelity

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The mitotic spindle is the key microtubular assembly necessary for proper chromosome segregation during mitosis. Additionally, a spindle matrix has long been proposed to support this process, but its biochemical nature remained elusive. By combining live-cell imaging with laser microsurgery, fluorescence recovery after photobleaching and fluorescence correlation spectroscopy in *Drosophila* S2 cells, we uncovered the mechanism that underlies accumulation of molecules to the spindle matrix. Supported by mathematical modelling, we demonstrate that a membrane system that surrounds the spindle region causes spatio-temporal differences in molecular crowding states that are sufficient to drive accumulation of Megator, a bona fide matrix protein, but also soluble tubulin and Mad2 in the spindle region. Overall, our findings reveal that cytoplasmic compartmentalization which also persists during 'open' mitosis promotes mitotic fidelity by spatially confining key processes such as spindle assembly.

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Azurin: a therapeutic protein that interferes with oncogenic signaling and blocks tumor progression

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Azurin is a small protein secreted by the bacterium Pseudomonas aeruginosa and, in the last years, it has been studied as a new anti-cancer therapeutic agent. Our group has shown that azurin is capable to block invasion of breast cancer cells, being this effect dependent on the overexpression of P-cadherin (Bernardes *et al.* 2013), which is a transmembrane protein with an important role for a subset of breast cancers. Actually, P-cadherin is a poor prognostic factor, a marker of breast cancer stem cells and an inducer of cell migration and invasiveness (Ribeiro *et al.* 2010; Vieira *et al.* 2012). Interestingly, azurin was able to impair all these effects, as well as the signaling pathway activated by P-cadherin, which mediates FAK/Src phosphorylation (Bernardes *et al.*, 2013).

We have further performed a genome-wide expression analysis of azurin-treated cells, in order to find new signaling pathways regulated by this peptide. Azurin significantly up-regulated endocytic processes, concomitantly with an induced decrease in the expression of cell surface receptors and associated signaling, and decreased adhesion to the extracellular matrix (ECM). These observations were confirmed by the decrease of integrin subunit receptors (α6, β1 and β4 integrin subunits) and a decrease in the ability to form mammospheres, an important parameter of cancer therapy resistance (Bernardes *et al.*, 2014).

Based on these results, we hypothesized that azurin exerts its cellular effects through lipid raft regions, altering the communication of cancer cells with their surrounding microenvironment, which has important consequences at several downstream signaling pathways. Therefore, by disrupting lipid rafts, which harbor a variety of signaling components such as integrin subunits or growth factor receptors, this new therapeutic peptide may target an important subset of cancer cells, such as cancer stem cells, alone or in combination with other therapies, improving the clinical outcome.

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CANCER | POSTER 55

HAPPY: Health Awareness and Prevention Personalized for You

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It is estimated that, by the year 2030, cancer will affect more than 26 million people worldwide and over 17 million will die from this disease. Tobacco and alcohol consumption, excessive exposure to the sun and lack of physical exercise are important risk factors for cancer. In fact, more than half of cancer cases are due to wrong behavioral options; if everyone adopted a healthier lifestyle, cancer incidence would fall dramatically.

Several studies have shown that the Portuguese population lacks cancer prevention knowledge and has an overall unhealthy lifestyle. This reality needs to be changed if we want to reduce cancer incidence and mortality. Information campaigns are critical to raise cancer awareness but they simply are not enough to promote behavior change. Tobacco consumption provides the perfect example: despite all the warnings and campaigns designed to promote smoking cessation, many people continue to smoke.

According to Fogg, behavioral changes occur when three elements converge in a given moment: Motivation, Ability and Trigger. If one of these three elements is missing, the change will not occur. This model clearly points out that motivation alone is not enough to induce a new behavior; the target behavior has to be simple enough to be performed by that person and a trigger has to be present to remind that person to perform that behavior. Fogg defines trigger as "something that tells people to perform a behavior now". An effective trigger will remind and instigate people to perform the target behavior. Mobile phones can be very useful in this sense: with mobile phones it is possible to persuade individuals to change their behavior by delivering the right trigger in the right moment.

The main purpose of this project is to research and develop a cancer prevention strategy, using mobile devices, capable of inducing behavioral changes on individuals. To achieve this purpose, a cancer prevention mobile application named HAPPY (Health Awareness and Prevention Personalized for You) is being developed. HAPPY includes the following major characteristics:

Personalization: personalized cancer prevention messages will be sent to users across time. The messages will be tailored according to the user's characteristics and will take into account the users context (location, time of day, time of year, weather);

Graphic feedback: users will monitor their behavior and assess behavior change. It is mainly a self-assessment and motivation tool.

Motivation elements: competition between peers (in form of weekly challenges) and motivational boosts (in form of achievements) will be embedded in the application.

HAPPY is currently in the prototype stage and is ready to be field-tested in a pilot study.

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Feedback control of chromosome separation by a midzone Aurora B gradient

Olga Afonso
IBMC

Accurate chromosome segregation during mitosis requires the physical separation of sister chromatids prior to nuclear envelope reassembly (NER). However, how these two processes are coordinated remains unknown. Using live-cell imaging, RNAi, pharmacological inhibitions and laser microsurgery in *Drosophila* S2 cells, we identified a conserved feedback control mechanism that delays chromosome decondensation and NER in response to incomplete chromosome separation during anaphase. A midzone-associated Aurora B gradient was found to monitor chromosome position along the division axis and prevent premature chromosome decondensation by retaining Condensin I. PP1/PP2A phosphatases counteract this gradient to trigger chromosome decondensation and NER. Thus, the Aurora B gradient appears to mediate a surveillance mechanism that prevents chromosome decondensation and NER until effective separation of sister chromatids. This promotes the correction and re-integration of lagging chromosomes in the main nuclei prior to completion of NER.

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Expression of Iron-related Proteins Suggests an Additional Role for Stromal Inflammatory Cells in Breast Cancer

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Iron is an essential element for several cellular processes. Although iron homeostasis in breast cancer epithelial cells has already been addressed, the role of stromal inflammatory tumor players in this respect remains unknown. Given the fact that circulating leukocytes are capable of taking up iron and exporting it in some conditions we hypothesize that stromal inflammatory cells, by constituting a potential iron delivery system, may have an important role in breast cancer (BCa) progression. Hepcidin, ferroportin, transferrin receptor 1 (TFR1) and ferritin expression were assessed by immunohistochemistry in a maximum of 325 tissue microarray spots from 146 primary tumors (59 breast reduction specimens, 18 ductal carcinomas *in situ* and 69 invasive ductal carcinomas) and in 24 axillary lymph nodes (12 metastized and 12 non-metastized). Tumor BCa cells present higher expression of hepcidin and TFR1 and lower expression of ferritin than mastectomy breast epithelial cells, suggesting an 'iron-utilization' phenotype, coincident with a proliferative status. On the other hand, tumor-associated lymphocytes and macrophages display an 'iron-donor' phenotype, as shown by higher ferroportin and ferritin expression. The observation of higher ferroportin and ferritin expression in pre-invasive stages suggests that these cells may play an additional role as mediators of tumor progression by acting as a local 'iron-delivery system'. This is further supported by the fact that TFR1 expression in lymphocytes and macrophages is positively correlated with tumor size and a higher expression of ferroportin and ferritin is observed in BCa metastized lymph nodes. Moreover, the expression of iron metabolism proteins, not only in epithelial cells, but also in stromal inflammatory cells correlates with classical biomarkers of BCa prognosis. In summary, we demonstrate that lymphocytes and macrophages in breast cancer present an 'iron-donor' profile that may contribute to the tumor cells nutrition, particularly in stages where the iron supply might be critical to invasion.

keywords: iron homeostasis, tumor microenvironment, lymphocytes, macrophages, 'iron-utilization' phenotype, 'iron-donor' phenotype

Hereditary Diffuse Gastric Cancer – How to survive the loss of adhesion

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Gastric cancer (GC) remains a major concern as the second leading cause of cancer-related mortality worldwide. Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant cancer syndrome caused by germline mutations in the E-cadherin encoding gene, *CDH1* (Guilford, 1998; van Roy, 2008). What is striking is that *CDH1* germline mutations confer more than 80% lifetime risk to specifically develop gastric cancer, despite that E-cadherin is expressed in all epithelial tissues (Carneiro, 2012). More so, the fact that stomachs of germline *CDH1* mutation carriers present up to several hundred foci of diffuse gastric cancer suggests that early HDGCs occur more due to the inherent characteristics of the tissue rather than to widespread mutations of the genome (Humar and Guilford, 2009).

We hypothesize that *CDH1* biallelic inactivation is only tolerated in gastric epithelium and not in other epithelia due to a favourable gastric-specific expression program that allows cell-autonomous survival.

Accordingly, we evaluated the expression profiles of a series of candidate stomach-specific genes and their functional significance in resistance to apoptosis induction by staurosporine upon loss of E-cadherin. Our data supports a role for S100P and CTSE in a gastric-specific molecular program that may ultimately encompass oncogenic potential. Furthermore, through a proteomic profiler approach, we observed a striking upregulation of anti-apoptotic proteins upon *CDH1* downregulation in gastric epithelium. Specifically, we postulate that inhibitor of apoptosis (IAP) proteins, such as XIAP and survivin, as well as Bcl-2 family members may be part of a survival program underlying gastric cancer development upon E-cadherin loss.

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CANCER | POSTER 59

Obesity, Melanoma and Radiation Therapy Susceptibility

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Background: Obesity, favored by the modern lifestyle, acquired epidemic proportions nowadays. Obesity involvement in melanoma etiology has been recognized, but the implicated mechanisms remain unclear. Although historically melanoma was considered a relatively radioresistant tumor, radiation therapy (RT) can be a useful treatment option for patients with melanoma in some settings. High visceral adiposity complicates the delivery of radiation therapy in obese patients. Therefore, we proposed to investigate the influence of distinct fat depots secretome in malignant melanocytes radiosensitivity.

Methods: Rat visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) fragments, 3T3-L1 pre-adipocytes and B16F10 melanocytes were cultured in DMEM. 3T3-L1 pre-adipocytes were differentiated into mature adipocytes using an established cocktail of 500 µM 3-isobutyl-1-methylxanthine, 250 nM dexamethasone and 10 µg/mL insulin. Subsequently, B16F10 cells were irradiated with a total dose of 2Gy and treated with conditioned medium (CM) obtained from the adipose tissue fragments or 3T3-L1 cultures for a 12 hours period. Later on, B16F10 cells viability, catalase activity and total antioxidant status (TAS) were accessed.

Results: After irradiation, B16F10 cells shown a 10% decrease in their viability, conversely when cultured with 3T3-L1 CM the malignant melanocytes cells increased their metabolic activity by 25% whether irradiated or not (*p<0,05; n=6). Both the secretomes of VAT and differentiated 3T3-L1 cells had an higher TAS, but it was the CM from SAT fragments that showed the highest concentration of antioxidants (*p<0,05; n=6). After irradiation, the TAS of melanocytes treated with SAT, VAT or 3T3-L1 CM was significantly higher than control (*p<0,05; n=6). Although no significant difference was observed between the different CM treatments, the total amount of antioxidants was continuously lower when cells where exposed to ionizing radiation (*p<0,05; n=12). Catalase activity was significantly lower in B16 cells treated with all CM, yet SAT CM treated cells showed the lowest expression of this enzyme - 6 fold decrease (*p<0,05; n=6)

Conclusion: Cell survival response and (anti)oxidant status are good indicators of RT susceptibility. Our results shown an increased metabolic activity of irradiated melanocytes when treated with adipocytes CM. Concomitantly, in the present of adipocyte secretomes, melanocytes had higher antioxidant capacity. The preliminary results obtained in the present study shown that adipose tissue secretome enhances melanocytes radioresistency.

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Synthesis, screening and evaluation of the activity of new p53 activators in human tumour cell lines

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Strategies to increase p53 activity are of major interest for cancer therapy, considering the central role of this tumour suppressor protein in the regulation of DNA damage response, cell cycle arrest and apoptosis [1-3]. The activation of *wt* p53 and/or of its targets could result in increased cell cycle arrest and cell death, ultimately abrogating tumour development. The aim of the present work was to synthesize new p53 activators and further confirm their activity in a yeast p53 assay and in human tumour cell lines.

A series of chalcones was synthesized by Claisen-Schmidt condensation of a 2'-hidroxyacetophenone with appropriately substituted benzaldehydes by microwave irradiation. Using the yeast p53 assay, this series of chalcones was screened for p53 activity, which allowed the identification of eight potent p53 activators.

These chalcones were selected to be further studied in a panel of four human tumour cell lines in order to confirm their *in vitro* cell growth inhibitory activity. The mechanism of action of the two most potent compounds was further investigated in the non-small cell lung cancer cell line NCI-H460 (in which the compounds were quite potent). Results confirmed that both compounds reduced NCI-H460 viable cell number and cellular proliferation. In addition, alterations in the cell cycle profile and/or induction of apoptosis were observed following treatment with the compounds. Moreover, these compounds were shown to increase the cellular expression levels of *wt* p53 and of its transcriptional target p21, further indicating that they are p53 activators.

In conclusion, the present work describes the synthesis of eight new chalcones. Among the synthesized compounds, two were found to be p53 activators when using a yeast p53 assay. Both compounds increase p53 and p21 expression levels in human tumour cell lines. Additional studies are needed to prove the potential of these molecules in human tumour xenograph models in mice.

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CANCER | POSTER 61

Screening the zebrafish for new genes required for vertebrate pancreas development

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Introduction: The development of the vertebrate pancreas is a complex process that includes regionalization, cell differentiation, and morphogenesis. Studies in zebrafish are contributing to uncover the genes and their associated roles required for this biological process. Previously, a genetic screen was designed in our lab to visualize and interrupt gene's regulatory landscapes in vertebrates, based on the Expression Disruption (ED) vector, an enhancer trap vector with strong mutagenic capacity for disconnecting cis-regulatory elements from targeted genes. Using this vector, a tissue-specific screen resulted in zebrafish ED lines showing reporter gene expression in the pancreas and we further identified a new set of genes expressed dynamically during pancreas development. Some of these genes were itag5, an integrin alpha 5; *ptrfb*, a cavin-1 required for caveolae biogenesis; *cdh2*, gene encoding N-cadherin and *bcas2*, a breast cancer amplified sequence 2. We aim to dissect the roles of these new genes in pancreas development and understand their requirement in the genetic networks of this organ.

Material and methods: To approach gene function, we used morpholinos and site-directed mutagenesis (Crispr-Cas9). To assess the function of these genes in pancreas development and function we performed in situ hybridization with digoxigenin labelled antisense RNA probes and immunofluorescence assays using different molecular pancreatic markers.

Results: In these preliminary assays we observed that the area of insulin expression is significantly reduced when we knockdown *ptrfb*, *cdh2* and *bcas2*. However, we did not observed a similar reduction when impairing the activity of the gene itga5. Immunofluorescence for the progenitor cell marker Pdx1 and the regulator of islet cell differentiation Nkx6.1 revealed that the knockdown of ptrfb also results in a reduced number of these cell types.

Conclusions: Our data suggest that the genes *ptrfb*, *cdh2* and *bcas2* may have a putative function in the differentiation of beta-cells.

CANCER | POSTER 62

Evaluating the role of *cis*-regulatory elements in pancreatic cancer using a zebrafish model

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Pancreatic carcinoma (PC) is a highly aggressive malignant disease with very poor prognosis, and a 5-year survival of less than 5%1. Familial predisposition for PC constitutes about 10% of the patients, but exonic germline mutations identified so far account for less than 20%, thus the underlying genetic predisposition remains unknown in most cases[2]. Genome-wide association studies (GWAS) have been providing crucial data on single nucleotide polymorphisms (SNPs) constituting potential risk alleles for PC, often localized at non-coding regions[3]. Mutations in non-coding regions, particularly cis-regulatory elements (CREs), may have a disease-causing effect, as it has been associated with beta-thalassemia, hemophilia, atherosclerosis[4] or cancer[5]. Zebrafish has been used as a model of PC and it has been demonstrated that genetic regulators of exocrine pancreatic development in zebrafish can be translated into potential clinical biomarkers and therapeutic targets in human PC[6].

The aim of this project is to disclose the role of *cis*-regulatory elements in PC. As a first approach we will unveil the array of CREs active in the zebrafish pancreas and find equivalent functional regions in human pancreas by combining ChIP-seq and 4C-Seq technologies with bioinformatics and data collected from GWAS on potential risk alleles for PC. From this screening, enhancers selected as active in adult pancreas and possibly constituting risk alleles for PC will be mutated in zebrafish and human cell lines and tested for their tumorigenic potential.

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Immunohistochemical molecular phenotypes of gastric cancer based on SOX2 and CDX2 predict patient outcome

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Background: Gastric cancer remains a serious health concern worldwide. Patients would greatly benefit from the discovery of new biomarkers that predict outcome more accurately and allow better treatment and follow-up decisions. Here, we sought to study the expression and prognostic value of the transcription factors SOX2 and CDX2 in gastric cancer.

Methods: SOX2, CDX2, MUC5AC and MUC2 expression were assessed in 201 gastric tumors by immunohistochemistry. SOX2 and CDX2 expression were crossed with clinicopathological and follow-up data to determine their impact on tumor behavior and outcome. Moreover, SOX2 locus copy number status was assessed in by FISH (N=21) and Copy Number Variation Assay (N=62).

Results: SOX2 was expressed in 52% of the gastric tumors and was significantly associated with male gender, T stage and N stage. Moreover, SOX2 expression predicted poorer patient survival, and the combination with CDX2 defined two molecular phenotypes, SOX2+CDX2- versus SOX2-CDX2+, that predict the worst and the best long-term patients' outcome. These profiles combined with clinicopathological parameters stratify patients' prognosis with intestinal and expanding tumors and in those without signs of venous invasion. Finally, SOX2 locus copy number gains were found in 93% of the samples reaching the amplification threshold in 14% and significantly associating with protein expression.

Conclusions: We showed, for the first time, that SOX2 combined with CDX2 expression profile in gastric cancer segregate patients into different prognostic groups, complementing the clinicopathological information. We further demonstrate a molecular mechanism for SOX2 expression in a subset of gastric cancer cases.

New HPLC-DAD Method for Oxidative Stress Evaluation

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Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidant levels in a cell, and the inability to detoxify the resulting reactive metabolites or to repair the damage caused by reactive species in a biological system.[1,2]

The determination of both reduced (GSH) and oxidized (GSSG) forms of glutathione is a major method for the evaluation of oxidative status. These levels allow the calculation of the ratio of [GSH]/[GSSG] that is among the most reliable parameters of such state [2,3].

Chromatographic methods allow identification and quantification of multiple substances. The present work presents a new chromatographic method, based on an acidic aqueous mobile phase and a Diode Array Detector, avoiding the organic solvent usage. The developed method allowed the simultaneous quantification GSH and GSSG forms and proved to be reliable, since the values obtained for total amount of glutathione in blood samples were in agreement with the same values obtained by a commercial kit assay method. Moreover, the new method is quick, and a run takes 15 minutes to perform. Also, sample preparation is fast and the cost of the analysis is significantly less than the amount expended in an analysis using a kit assay.

We emphasize the fact that this method runs in an acidic aqueous isocratic eluent, organic solvent free, which carries less environmental issues and allows the mobile phase recycling.

In conclusion, the new method developed is presented as extremely advantageous from every point of view and can be expected to have a major impact on the study, diagnosis and treatment of various diseases oxidative stress related[4].

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Bioactivity of quinoxaline 1,4-dioxide derivatives

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There are *in vitro* and *in vivo* studies revealing the proliferative and toxic effect of antibiotics[1,2], namely β-lactams and quinolones, in both human and animal cell lines. The selectivity of bacterial cells to antibiotics is not evident and the effects on eukaryotic cells must be elucidated. There may be implications on tissue regeneration, as in orthopedic patients, or in tumor cells, that have altered metabolism[2] by exposure to xenobiotics. Eukariotic cell metabolic rate alteration is an important factor to consider when exploiting new therapeutics in order to prevent possible critical side effects.

Quinoxaline is a chemical compound that presents a structure similar to quinolone antibiotics. The present work reports the study of the biologic activity of quinoxaline N,N-dioxide and four derivatives:quinoxaline-1,4-dioxide(QNX),2-methylquinoxaline-1,4-dioxide (2MQNX), 2-amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX), 3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC) and 2-hydroxyphenazine-N,N-dioxide (2HF).

Antimicrobial activity was tested against Gram-positive and Gram-negative bacterial strains and yeast strains[3].

In the present work, toxicity and anti-tumor activity was tested against seven cell lines, two fibroblast-like cell lines, one osteoblast cell line and four tumor cell lines.

The results obtained for cell lines indicate low toxicity and suggest no significant toxic effects on fibroblast-like cell lines and on osteoblast promoting cells It was observed a proliferative effect on one of the fibroblast-like cell lines. Moreover, these findings suggest that, regarding to other quinolones, such as fluoroquinolone, this group of quinoxaline derivatives may present an advantage, in terms of the adverse effects on the bone. There is no significant proliferative influence in tumor cell lines tested. On contrary, results show a negative influence on cell growth, suggesting some anti-tumoral effect.

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Identification of a site-specific modification of E-cadherin N-glycans with key roles in its critical regulation in gastric cancer

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E-cadherin is a cell adhesion molecule and the major component of the adherens-junctions in epithelial cells, playing a pivotal role in suppression of cancer. E-cadherin downregulation is a key feature of the majority of invasive gastric carcinomas. Recently, glycosylation modifications have been described as a key molecular mechanism underlying the dysregulation of E-cadherin functions in gastric cancer cells (1-3). We have recently demonstrated that E-cadherin glycosylation mediated by GnT-V induces a deleterious effect on E-cadherin biological functions with destabilization of adherens junctions, being associated with tumour cell invasion and progression (4). We have also demonstrated that gastric cancer patients displaying E-cadherin loss of function without genetic/epigenetic alterations exhibit a significant increased glycosylation with the β1,6 GlcNAc branched N-glycan structures, which was found to underlie its functional impairment in gastric cancer (4). It remains to be further determined the site-specific modification of the branched N-glycans on E-cadherin, catalyzed by GnT-V. E-cadherin ectodomain has four potential N-glycosylation sites, and its occupancy and functional relevance is far from being fully elucidated.

Combining *in silico* bioinformatics analysis and site-directed mutagenesis of the four potential N-glycosylation sites of E-cadherin followed by enzymatic digestion approaches we demonstrated that E-cadherin is modified with complex-type N-glycans at Asn-554 and high mannose/hybrid- type N-glycans at Asn-633 whereas Asn-566 and Asn-618 are not likely to be N-glycosylated. We have also identified the selected site modified with the deleterious β1,6 GlcNAc branched structures catalyzed by GnT-V affecting the biological functions of E-cadherin (cellular expression; E-cadherin-mediated cell-cell adhesion and dimerization; assembly of adherens junctions) in a gastric cancer context.

In this study we clarified the structural and functional mapping of E-cadherin N-glycans and its impact in cancer cell biology. The identification of the site-specific modification of E-cadherin with the deleterious N-glycans structures opens the opportunity for the development of novel diagnostic and therapeutic strategies with potential translational clinical applications in the cancer setting.

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Morphofunctional modulation of GC1a cell line by oocyte factors

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The success of assisted reproductive techniques is partly dictated by the oocyte quality, achieved during its development. A major contribution for oocyte quality is provided by the follicular microenvironment that includes the oocyte adjacent cells. These comprise granulosa cells (GCs) that multiply along oocyte growth and development; which differentiate into two structurally and functionally distinct types: the mural GCs and cumulus cells (CCs). In this settings, the oocyte is an indirect regulator of itself. In fact, it produces the oocyte-secreted factors (OSFs), which are members of TGF-β superfamily growth factors able to modulate follicular cells functions and survival that, in turn, also modulate oocyte growth.

In order to increase our understanding on the role of OSFs in GCs, and also aiming to uncover molecules with potential properties of clinical biomarkers, we employed an immortalized human granulosa cell line, the GC1a. Apart from its better characterization, its use is likely to circumvent considerably the lesser progress attained when primary culture of human granulosa cells are employed instead.

In this work, we aimed to characterize the dynamic process between GCs and follicular microenvironment. Early and late cell passage numbers were elected for experiments performed by adding OSFs and FSH to the media and comparing with the controls.

We have reported for the first time in this cell line the presence of OSFs transcripts, namely GDF9, and also the OSFs receptors, such as BMPRI, BMPRII, TGFβRI. We also demonstrated that OSFs and FSH do not affect cell proliferation and cell cycle progression, in GC1a cell line.

Our data also suggest that the increase of passage number has an effect in GC1a cell line, it has a role in cell morphology, inducing cellular differentiation; apart from it is able to modulate ERK1/2 and SMAD2 signaling, by OSFs and FSH stimulation, once in early passages FSH and GDF9 were able to activate ERK1/2 signaling; in late passages FSH is not capable to activate neither of them, however GDF9 strongly activates SMAD2 cascade.

The data as a whole suggest that this cell line can mimics CCs cells, regarding the presence of OSFs and their receptors and it morphofunctional response to the passage number and stimulation can have biological applicability.

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Establishment of a 3D model of EMT/MET-induction using molecularly-designed ECM-like hydrogels

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Epithelial to mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell to undergo biochemical changes that enable it to assume a mesenchymal cell phenotype. Therefore, EMT has been associated with a concomitant decrease of epithelial markers (e.g. E-cadherin) and increase of mesenchymal markers (e.g. Vimentin and α-SMA), as well as enhanced migratory and invasion capacity, resistance to apoptosis and increased production of extracellular matrix (ECM) components. EMT-cells may undergo a reverse process, mesenchymal to epithelial transition (MET), through which they are able to recover the epithelial phenotype. In some cases, EMT reversion may not be fully accomplished, giving rise to a metastable phenotype characterized by a simultaneous expression of epithelial and mesenchymal features. EMT has been shown to occur both under physiological settings, namely embryogenesis and wound healing, and in pathological situations, such as fibrosis. Several reports suggested that EMT/MET may also have a role during cancer progression and metastasis establishment. To elucidate this hypothesis, different EMT models have been implemented using traditional two-dimensional (2D) systems. However, such models lack the influence of a proper three-dimensional (3D) ECM-like structure that could better mimic the *in vivo* microenvironment, a major regulator of the EMT/MET program. Therefore, improved *in vitro* systems that better mimic the tumour microenvironment are needed in order to closely reproduce the EMT transcriptional program that occurs *in vivo*.

The central aim of work was the establishment of a 3D *in vitro* model of TGF-β1-driven EMT/MET induction in EpH4 epithelial cells cultured on an artificial alginate-based matrix with tuneable properties. In addition, we aimed to further characterize the 2D *in vitro* model of TGF-β1-driven EMT/MET induction previously established in the group, by assessing the stemness features of the EpH4 cells in different stages of the process, as well as their ability to produce matrix metalloproteinases (MMPs).

Our results demonstrated that the 2D EMT-derived cells have enhanced stem-like features (resulting from the enrichment in a $CD29^{+}/CD44^{+}$ subpopulation) and produce increased levels of MMP9. Regarding the 3D EMT/MET model, our findings showed a TGF-β1-mediated EMT program characterized by E-cadherin impairment and increased mesenchymal markers, both at RNA and protein level (e.g. Vimentin and α-SMA). Moreover, the removal of TGF-β1 stimulus enabled the establishment of a metastable phenotype with concomitant E-cadherin presence at cell membrane and α-SMA expression in the cell cytoplasm.

In conclusion, we created a 3D *in vitro* TGF-β1-mediated EMT/MET system that makes use of RGD-modified alginate hydrogels that provide a well-defined and tuneable environment. The insights gained with this work, may ultimate be useful for the establishment of a high-throughput system for the study of cancer progression and metastasis.

FoxM1 in aging and cancer

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Oncogenic transcription factor FOXM1 is over-expressed in the majority of human cancers (1). Emerging data suggest that targeting FOXM1 in mono- or combination therapy may have promising therapeutic benefits for the treatment of cancer (2). FOXM1 expression is associated with the proliferative capacity of the cell, consistently with its role in primarily driving the expression of G2/M specific genes, with associated phenotypic expression of mitotic defects and chromosome aberrations when defective (3). Using high-resolution live cell imaging, we found that human elderly dermal fibroblasts reproduce the mitotic defects of FOXM1 repression. In agreement, expression of FOXM1 and its downstream targets decreases progressively with chronological aging, suggesting it counters senescence. Importantly, we found that old fibroblasts are sensitized to cell death when treated with anti-mitotic drugs (such as taxanes and vinca alkaloids) commonly used in chemotherapy. We therefore both depleted FoxM1 in young cells and overexpressed a constitutively active FoxM1 in old cells, to follow their cell fate upon anti-mitotic drug treatment and determine if FoxM1 repression accounts for the increased sensitivity to anti-mitotics in aged cells. In addition, a combinatorial treatment using FOXM1 inhibition plus microtubule poisons is being tested for the pro-apoptotic efficacy against tumor cell lines overexpressing FoxM1. Our findings might uncover molecular mechanisms that could be explored therapeutically to both extend longevity and prevent aging diseases such as cancer.

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Keywords: FoxM1; aging; anti-mitotic drugs; cancer treatment

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Exploring New Strategies for Colorectal Cancer Therapy: a MAPK- and PI3K-Targeted Approach

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Colorectal cancer (CRC) is one of the leading causes of cancer mortality worldwide. It is often associated with activating mutations in KRAS, BRAF and PIK3CA leading to the deregulation of major signaling pathways as the RAS-RAF-MAPK and PI3K-PTEN-AKT. However, information is scarce regarding the molecular mechanisms underlying the survival of mutant CRC cells that are mostly resistant to the available therapies. At present, the alternative therapeutic option for patients with metastatic CRC (mCRC), in addition to the conventional therapies, involves the use of EGFR antibodies but only a small percentage of patients benefit from such therapies. Thus, it is urgent to unravel new strategies for CRC therapy and explore putative biomarkers predictive of therapy outcome.

Therefore, to elucidate the potential benefit of targeting the MAPK and PI3K signaling pathways in CRC patients, the effects of MEK1/2 and/or PI3K inhibition on cellular proliferation and survival were assessed in human CRC cells. More specifically, proliferation/viability, cell cycle and apoptosis were monitored in human CRC cell lines harboring genetic alterations in KRAS, BRAF and/or PI3K. Furthermore, the identification of key signaling molecules mediating MEK1/2 and PI3K inhibition was pursued.

Interestingly, targeted inhibition of MEK1/2 and PI3K modulated cell viability/proliferation, cell cycle and apoptosis differentially in human CRC cells with distinct mutational status for KRAS, BRAF and PI3K. Moreover, the results demonstrate a pivotal role for PI3K in the survival of CRC cells highlighting the interplay between the two signaling pathways. The results also demonstrate that the mechanisms underlying the effects of MAPK and PI3K inhibition can diverge depending on the cellular context, as targeted depletion of PI3K induced apoptosis or cell cycle arrest in distinct CRC cells. Furthermore, the analysis of a multitude of proteins by the use of antibody arrays revealed specific alterations in a wide spectrum of kinases and apoptosis-related proteins upon MEK1/2 and/or PI3K silencing.

Overall, this study demonstrates that specific inhibition of MAPK and/or PI3K pathways could provide an alternative approach for CRC patients. The identification of novel therapeutic strategies will undoubtedly have an impact in the clinical management and, eventually, on the overall survival rate of patients with mCRC.

Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis

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Exosomes are secreted by all cell types and contain proteins and nucleic acids. Here, we report that breast cancer associated exosomes contain microRNAs (miRNAs) associated with the RISC Loading Complex (RLC) and display cell-independent capacity to process precursor microRNAs (pre-miRNAs) into mature miRNAs. Pre-miRNAs, along with Dicer, AGO2, and TRBP, are present in exosomes of cancer cells. CD43 mediates the accumulation of Dicer specifically in cancer exosomes. Cancer exosomes mediate an efficient and rapid silencing of mRNAs to reprogram the target cell transcriptome. Exosomes derived from cells and sera of patients with breast cancer instigate non-tumorigenic epithelial cells to form tumors in a Dicer-dependent manner. This study identifies a mechanism whereby cancer cells impart an oncogenic field effect by manipulating the surrounding cells via exosomes. Presence of Dicer in exosomes may serve as biomarker for detection of cancer and offer opportunities for the development of exosomes based therapies.

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Cardiomyocytes: a signature of the ontogenic development

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Introduction: Among the cellular constituents of the myocardium, cardiomyocytes (CMs) are the structural foundation of the heart and their morphology and maturation are shaped by the increase in complexity and mechanical stress along heart development. Despite the pivotal role of CMs, its developmental history within myocardial own embryonic/fetal lifetime is still unknown. In the present work, we have performed a detailed evaluation of the proliferative subsets, with a focus on CMs during heart morphogenesis.

Materials & Methods: Hearts were isolated from C57BL/6 mice at embryonic and fetal stages. Hearts were either sequentially digested with collagenase for flow cytometry/sorting and *ex vivo* cell analysis; or processed for paraffin and gelatin inclusion for histochemistry and immunofluorescence, respectively. Transcriptional profile was obtained by qPCR.

Results: A detailed morphological analysis performed throughout cardiac morphogenesis allowed us to correlate alterations in CMs morphology with increased myocardial complexity and decreased proliferative activity by Ki67/pH3 expression. Interestingly, we have identified a novel surface marker (CD24) in cardiac field, which seems to identify a fraction of cycling CMs specifically located in the compact layer of myocardium in early developmental stages. Moreover, the frequency of $CD24^+$ cells is seen to decrease overtime becoming more restricted to the sub-epicardial zone in fetal hearts.

Discussion/Conclusion: The identification of a cell surface signature for immature CMs constitutes a promising tool to specifically isolate these cells and further characterize myocardium histogenesis. It is anticipated that this approach will highlight potential link(s) between cells contributing for cardiogenesis and cells engaged in neonatal myocardium regeneration following injury.

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Galectin-3 is a potential serum biomarker in canine mammary tumours

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Galectin-3 (Gal3) is a member of the family of carbohydrate binding proteins (β-galactoside-specific lectins) that has been implicated in multiple roles such as cell-cell and cell-ECM adhesion, angiogenesis promotion, cell proliferation and apoptosis resistance, the latter actions ultimately facilitating tumour progression and metastasis.

To explain the role of Gal3 in tumour progression and metastasis and to investigate the possibility to use serum Gal3 levels as cancer biomarker, Gal3 concentrations in serum samples of female dogs with malignant mammary tumours (MMT) were measured before and after the tumour resection and between females with and without metastatic disease.

The serum Gal3 levels before the surgery varied between 0 and 6.32 ng/mL (median, 1.13 ng/mL), after surgery between 0 and 9.93 ng/mL (median, 2.22 ng/mL) and three months later between 0 and 9.11 ng/mL (median, 1.48 ng/mL). Serum Gal3 levels one month after surgery are significantly higher than before the surgery (P=0.0058) and the median for Gal3 levels four months after surgery is lower than the median value for Gal3 levels one month after, but this difference was not statistically significant. Gal3 median values are generally higher in females with metastatic disease than with localized tumours, however this difference is only statistically significant for the serum samples collected seven (P=0.0007), ten (P=0.0061) and thirteen months (P=0.0052) after surgery.

Data obtained in this project allowed us to believe that the detection of increased serum Gal3 levels in certain patients with MMT may reflect some biological aspects related to metastatic process. Therefore, it is possible that an assay for detection of Gal3 levels in serum can be used to the diagnosis of metastatic disease. However more studies are necessary to determine the clinical value of these results: study replication including the comparison with healthy subjects (to assess normal Gal3 values in serum); include female dogs bearing benign tumours, to avoid the occurrence of flaws arising from cancer-induced inflammation; finally, more female dogs with metastatic disease are needed since the population involved in this study was quite small.

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Extracellular vesicles shed by multidrug resistant cells are bigger than their sensitive counterparts and have a cargo with a specific signature of proteins involved in their biogenesis

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Overexpression of drug efflux pumps such as P-glycoprotein (P-gp) can be a serious challenge to the efficient treatment of cancer, being a significant cause of multidrug resistance (MDR) [1]. This problem is aggravated by the fact that currently there are no validated means to diagnose and counteract MDR[2]. Extracellular vesicles (EVs) have been previously shown to be involved in the intercellular transfer of functional P-gp from MDR cells to drug-sensitive recipient cells [3]. In order to identify biomarkers of MDR, in the present work we compared EVs shed by MDR cells with the EVs shed by their drug-sensitive cellular counterparts. With that purpose, two pairs of MDR models were used (chronic myeloid leukemia and non-small cell lung cancer) and the EVs shed by those cells were characterized according to size, molecular profile of EVs markers and protein cargo. Results showed for the first time that EVs shed by MDR cells were more and bigger than their drug-sensitive counterparts. In addition, proteomic analysis (validated by Western Blot) of the protein cargo of these pairs of EVs identified different signatures of proteins known to be involved in the biogenesis of EVs. We suggest that the determination of size and signature of those proteins could be further explored as a biomarker to detect and predict MDR phenotype.

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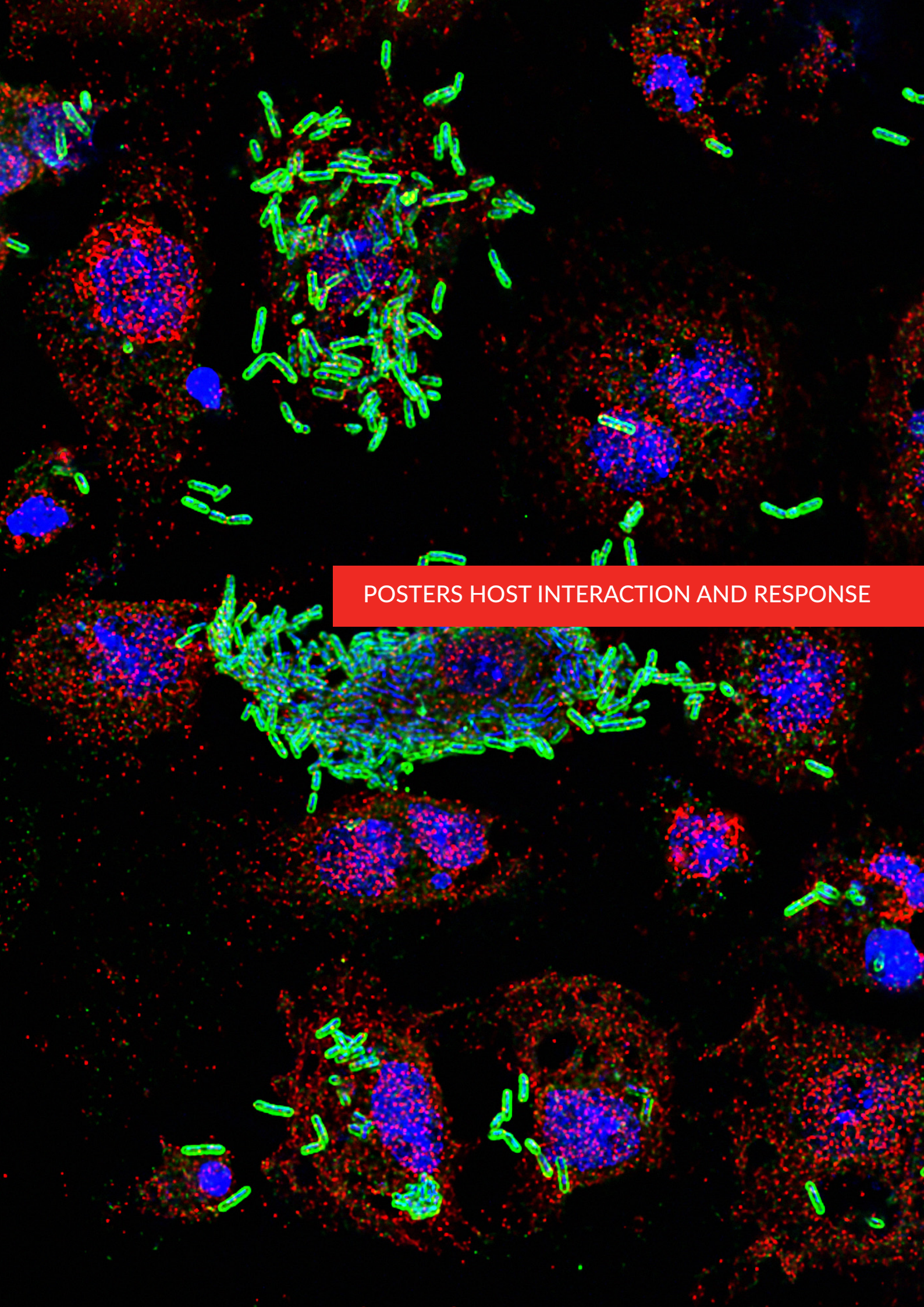

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A novel porous 3D silk fibroin/ nanohydroxyapatite hydrogel for bone regeneration

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The approach to tissue engineering involves regenerating tissue within suitable scaffold with the goal of implanting the constructed tissue at the target site. The regeneration of functional tissue requires a suitable microenvironment that closely mimics the host site for desired cellular responses, which is typically provided by 3D scaffold that act as an architectural template [1, 2]. Hydrogels are one category of scaffold that has been widely used in tissue engineering and regenerative medicine. They represent promising systems for the healing and regeneration of damaged tissues since they are highly permeable and facilitate the transport of nutrients and metabolites [3]. In bone tissue engineering, a biodegradable scaffold is a temporary template introduced at the defective site or lost bone to initiate bone tissue regeneration, while it shall gradually degrade and be replaced by newly formed bone tissue. The natural biopolymer silk fibroin (SF) is currently a subject of interest in bone tissue engineering and the inclusion of nanosized HA particles aggregates into biodegradable SF hydrogels should improve osteogenic outcomes. Therefore, in this work, we report the preparation and characterization of nanoHA and silk fibroin composite hydrogels. Composite hydrogels of *Bombyx mori* SF and nanoHA were fabricated by a new method using ethanol as gelling agent, where the nanoHA content varied from 10 (w/w)% to 30 (w/w)%. The structure and properties of the composite hydrogels were investigated by SEM, XRD, FTIR and compression modulus. The results showed that SF/nanoHA composite hydrogels presented a macro/ microporous structure with open interconnected pores, and the distribution of nanoHA into the composites was uniform. XRD and FTIR data showed that the silk fibroin in the composites was predominantly in a β- sheet crystalline structure (silk II) and both analyses confirm the existence of nanoHA particles in the composite. The mechanical results showed that the compression modulus of composite hydrogels was increased as the nanoHA concentration also increased.

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Fluorescent Bionanoprobes based on Quantum dot-Chitosan-O-Phospho-L-serine Conjugates for Labeling Human Bone Marrow Stromal Cells

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Bone replacement materials might be promising alternatives to autogenous bone transplants during the repair and reconstruction of human bone tissues. However, bone healing is a very complex process, and the role of phosphatidylserine (PS) and its moieties are not yet completely understood. In the present study, fluorescent quantum dots (QDs) functionalized with chitosan-O-phospho-L-serine (chi-OPS) conjugates have been synthesized and characterized while focusing on their potential applications as nanoprobes for labeling human bone marrow stromal cells (hBMSC). Essentially, chitosan was covalently linked to the peptide (O-phospho-L-serine, OPS) through the formation of amide bonds. In this sequence, these chi-OPS conjugates were utilized as direct capping ligands during CdS QDs (CdS/chi-OPS) biofunctionalization, which was achieved using a single-step process in an aqueous medium at room temperature. The core-shell nanostructures were characterized in detail by UV-visible spectroscopy (UV-Vis), photoluminescence spectroscopy (PL), atomic force microscopy (AFM), and transmission electron microscopy (TEM) with selected area electron diffraction (SEAD). The TEM images associated with the UV-vis optical absorption results indicated that ultra-small nanocrystals were formed with average diameters ranging from 2.2 to 2.8 nm. In addition, the PL results showed that the nanoconjugates exhibited “green” fluorescent activity under ultraviolet excitation. Cell viability was assessed *in vitro* via an MTT analysis, revealing that the bioconjugates were not cytotoxic after 3 days of incubation. Moreover, a quantitative flow cytometry (QFC) analysis and confocal fluorescence microscopy (CFA) were performed, verifying the fluorescence-labeling efficiency and the endocytosis of the bio-nanoprobes by hBMSC. In summary, innovative fluorescent conjugates were developed with properties for use as biomarkers when imaging and detecting bone tissue regeneration and metabolic events.

KEYWORDS: Nanomaterials; Bionanoprobes; Quantum dots; Bionanoconjugates; Nanotechnology; Bone Tissue Engineering.

Leishmania Infantum Modulates Host Macrophage Mitochondrial Metabolism by Hijacking the SIRT1-AMPK Axis

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Metabolic manipulation of host cells by intracellular pathogens is currently recognized to play an important role in the pathology of infection. Nevertheless, little information is available regarding mitochondrial energy metabolism in *Leishmania* infected macrophages. Here, we demonstrate that during *L. infantum* infection, macrophages switch from an early glycolytic metabolism to an oxidative phosphorylation, and this metabolic deviation requires SIRT1 and LKB1/AMPK. SIRT1 or LBK1 deficient macrophages infected with *L. infantum* failed to activate AMPK and up-regulate its targets such as Slc2a4 and Ppargc1a, which are essential for parasite growth. As a result, impairment of metabolic switch caused by SIRT1 or AMPK deficiency reduces parasite load *in vitro* and *in vivo*. Overall, our work demonstrates the importance of SIRT1 and AMPK energetic sensors for parasite intracellular survival and proliferation, highlighting the modulation of these proteins as potential therapeutic targets for the treatment of leishmaniasis.

Chitosan microparticles - a solution to eradicate *H. pylori* from gastric mucosa?

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Introduction: *Helicobacter pylori* (*H. pylori*) colonize the gastric mucosa of over 50% of the world's population, causing gastric disorders from gastritis to gastric cancer [1]. Current treatments rely on antibiotic-based therapies, but are inefficient in 20% of the cases, leaving ~140 million patients worldwide without alternative treatment [2]. Chitosan microspheres (Ch Mic) have recently been proposed as H. pylori-binding system, mainly due to their antimicrobial/mucoadhesive properties [3,4]. After oral administration, Mic should bind *H. pylori* present in the mucosa, removing them through the gastrointestinal tract. Ch Mic with 170µm can adhere to *H. pylori* [4], but are unable to enter into the human gastric foveolae which are ~70µm wide. This work aims to produce smaller Ch Mic and evaluate their size, morphology and interaction with gastric mucosa and *H. pylori* using different bioimaging techniques.

Materials & Methods: Chitosan microparticles (Mic) were produced by ionotropic gelation using an aerodynamically assisted spraying system, varying parameters such as Ch concentration, degree of acetylation and flow rate, air pressure and nozzle diameter. Mic were then crosslinked with genipin (10mM, 45min) to avoid dissolution in gastric pH, followed by lyophilization. Size, morphology, area and circularity features of Mic were evaluated using Mastersizer (MS), IN Cell Analyzer high-throughput microscope (ICA) and ImageStream imaging flow cytometer (IS). Ability of Mic to enter into mice and human gastric foveolae was evaluated by incubating Mic with fresh stomach samples at pH 6.0. Gastric mucosa was labeled with CellMask™ Deep Red plasma membrane stain and assessed with a confocal microscope (CM). Mic's ability to adhere *H. pylori* after incubating Mic with FITC-labeled *H. pylori* at pH 6.0 was assessed by CM and IS.

Results and Discussion: The optimization process of Mic production revealed 525mBar, 0.25mm nozzle, Ch concentration of 0.5%(w/v) with DA of 6% and 0.5mL/min flow rate, as the optimal conditions to obtain Mic with the required size. MS equipment provided, by laser diffraction, the size distribution of Mic, revealing that 50% of them presented a diameter <55.2µm. After lyophilization, ICA showed, in an automated way, the average diameter (~18µm), maximum chord (~43µm), area (~786µm2) and form factor (0.72) of Mic, revealing their irregular morphology. Penetration studies analyzed by CM revealed the presence of Mic in different depths of the gastric mucosa, thus indicating their ability to enter into mice and human foveolae. Moreover, CM and IS revealed FITC-labeled *H. pylori* ability to adhere throughout the surface, but not inside the Ch Mic.

Conclusion: Chitosan microparticles with 18µm average diameter were successfully produced and are able to penetrate mice and human gastric mucosa (*ex vivo*) as well as to adhere *H. pylori* (*in vitro*). CM, ICA and IS were revealed as particularly relevant bioimaging techniques in this study.

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Long-term protection against neosporosis in mice intranasally immunized with *Neospora caninum* membrane antigen extracts

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Neospora caninum is an intracellular protozoan causative of repeated abortions in cattle, responsible for economic losses estimated above US $1.3 billion/annum. No vaccine or other effective method is currently available to manage neosporosis, the disease caused by this parasite. Recently, we demonstrated the efficacy of intranasal immunization by using *N. caninum* membrane protein extracts (NcMP) plus CpG adjuvant in conferring protection against intragastrically (i.g.) established murine neosporosis (Ferreirinha *et al.*, Immunology, 2014). Here, we evaluated whether this protection could still be observed in the long–term. Mice immunized twice (3 wk apart) with 30 µg NcMP plus 10 µg CpG were infected i.g. with 5×10^7 tachyzoites 18 wk upon the last immunization. Controls were non-immunized or sham-immunized with CpG alone. Significant protection was observed in the immunized mice as parasite DNA in the brain was detected in only 1/9 mice vs 6/9 and 8/10 in non-immunized and sham-immunized mice, respectively, 7 days upon infection. The effectiveness of mucosal immunization was monitored by assessing antigen-specific vaginal IgA and serum IgG1 and IgG2a levels, that were found elevated all along the experiment, with an IgG2a/IgG1 ratio > 1. Specific intestinal IgA levels were also found elevated upon infection in the immunized mice. Spleen and mesenteric lymph node cells collected from immunized mice 17 wk after the last immunization (1 wk prior to infection) presented a higher *in vitro* production of upon recall antigen stimulation than control counterparts. However, this higher IFN-γ production in the immunized mice was not observed when this analysis was performed 1 wk after infection. Our results show that intranasal immunization with NcMP plus CpG induces a sustained systemic and mucosal immune response and confers a long-term protective effect against neosporosis established through the gastrointestinal tract.

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Docosahexaenoic acid delivery system for *H. pylori* infection treatment

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Introduction: *Helicobacter pylori* is a gram-negative, microaerophilic and spiral-shaped bacterium that colonizes the gastric mucosa of over 50% of the World's population [1]. Persistent infection of H. pylori has been associated with increased risk for development of gastric cancer. *H. pylori* infection recommended treatment is based in a combination of at least two antibiotics and a proton pump inhibitor [2]. However, this therapy fails in 20% of patients especially due to bacterial resistance [3].

Correia *et al* demonstrated that a lipophilic compound, docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid present in fish oil, decreases *H. pylori* growth *in vitro* in a dose-dependent way and inhibits mice gastric colonization *in vivo*. However, up to 40% of infected mice, following DHA treatment, are still colonized by bacteria [4]. This might be explained by the low penetration of DHA through the stomach mucus layer, which leads to an insufficient concentration of DHA to eradicate *H. pylori* at the infection site. The aim of this work is the encapsulation of DHA to create a gastric delivery system to improve DHA efficacy against *H. pylori*.

Materials & Methods: DHA was incorporated into nanostructured lipid carriers (NLC), submicron colloidal carriers composed of biodegradable and biocompatible lipids recognized for the incorporation of lipophilic and poorly water-soluble drugs [5]. NCLs with different DHA concentrations (0, 1, 2 and 2.5% v/v) were produced by hot homogenization technique using cetyl palmitate (NLC-CP) or precirol ATO5 (NLC-P) as solid lipids, migliol-812 as the liquid lipid and polysorbate 60 as the stabilizer. DHA-NLCs were characterized by dynamic light scattering (DLS) and DHA incorporation was quantified using spectrophotometry. The effects of DHA-NLCs on *H. pylori* J99 growth were analyzed *in vitro* using different concentrations of NLCs-P (50, 100 and 500 µM) prepared with 2% v/v DHA . Number of viable bacteria was determined by colonies forming unit (CFUs) counting.

Results: All produced NLCs nanoparticles have a size of 150-250 nm, negative zeta potential (~ -30 mV) and a DHA entrapment higher than 50%. Preliminary results demonstrated that NLCs-P, even without DHA, were able to decrease/inhibit *H. pylori* growth in a concentration dependent way. All DHA-NLCs demonstrated a bactericidal effect on *H. pylori* J99 in opposite to DHA in solution that was only bactericidal at concentrations around 500 µM.

Conclusion: NLCs nanoparticles can be used to encapsulate DHA improving its bactericidal effect on *H. pylori*.

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Development and characterization of poly (ethylene oxide)-modified nanoparticles containing saquinavir for the prevention of rectal HIV transmission

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Background: Microbicides are gaining substantial ground as a potential tool for the prevention of sexual HIV transmission. The development of rectal microbicides in particular is highly desirable due to the prevalence of unprotected anal intercourse in both homosexual and heterosexual individuals and the higher risk of transmission via the rectal route[1]. Despite relevant progress in the field, several issues related with the formulation of products developed so far have jeopardized the development of an effective rectal microbicide[2]. Recently, nanotechnology-based systems emerged as a promising strategy to circumvent problems found with conventional dosage forms commonly used in the formulation of microbicide compounds[3]. In particular, mucus penetrating nanoparticles (NPs) obtained by dense poly(ethylene oxide) (PEO)-coating may be interesting platforms for the rectal delivery of antiretroviral drugs to prevent HIV transmission since they show the ability to transpose the mucus barrier more efficiently than mucoadhesive ones. Consequently, NPs can eventually reach the underlying epithelium and deliver active payloads to immune cells susceptible to the virus[4]. The purpose of this work is to develop and characterize poly (lactic-co-glycolic acid) (PLGA) NPs coated with PEO and intended to be used for the rectal delivery of the anti-HIV protease inhibitor saquinavir (SQV).

Methods: PLGA nanoparticles containing the antiretroviral SQV were prepared by nanoprecipitation method[5] and further incubated in aqueous solution of the triblock copolymer poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO; poloxomer 407) used in different concentrations, ranging from 0.5% to 4% (w/v). Produced PEO-PLGA NPs were characterized regarding size and size distribution (Dynamic Light Scattering), and zeta potential (Laser Doppler Anemometer). Surface morphology was assessed by scanning electron microscopy. The increasing presence of PEO chains at the NPs surface due to poloxamer physical adsorption was assumed by the variation of zeta potential. Further, association efficiency percentage (AE%) and drug loading percentage (DL%) were accessed by a HPLC-UV method.

Results: SQV-loaded PEO-PLGA nanoparticles presented spherical shape with average size ranging from 150 to 300 nm and relatively homogeneous size distribution for all formulations (polydispersity index ≤ 0,300). Uncoated NPs presented zeta potential values around -25 mV but increased with poloxamer coating in a concentration-dependent fashion to values in the range of -20 mV to -3 mV. Less negative zeta potential values were obtained with increasing poloxamer concentration thus suggesting variable poloxamer adsorption at the surface of NPs. Near neutral surface charge was obtained when using poloxamer concentrations of 2-4%. These last NPs may prove useful in rectal microbicide drug delivery since it has been reported that NPs densely coated with PEO are able to rapidly penetrate mucus layers [4, 6]. The DL% of the SQV was approximately 3% and the AE% close to 60% for all formulations.

Conclusion: PEO-modified PLGA NPs were successfully obtained by the hereby proposed methodology, presenting the potential to be used as mucus penetrating nanocarriers for the delivery of SQV in the context of rectal microbicides.

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A three-dimensional cell-based intestinal *in vitro* model – an earlier stage tool to explore intestinal absorption

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Despite the huge palette of *in vitro* intestinal models currently available, considerable improvements are still a necessity for the drug development industry. 'Petri-dish'-based models provide limited potential to predict cellular responses of real organisms as they do not replicate the architecture and the mechanisms representative of a living tissue [1]. In contrast, 3D culture models encompass a great potential to build up a bridge between the cell culture models and *in vivo* animal models [2]. The present study extended a traditional intestinal model (Caco-2 cell monolayer) to the deeper layers of the small intestine, in particular the intestinal mucosa that includes a broad variety of stromal cells. Fundamentally, this model meant to dissect the stromal and epithelial interactions in the intestinal epithelium and evaluate the permeation of a protein drug, insulin, using a 3D approach. The model comprises CC18-Co intestinal myofibroblasts embedded in a Matrigel® matrix, Caco-2 and HT29-MTX epithelial cells and Raji B lymphocytes. Raji B lymphocytes trigger the conversion of Caco-2 cells into M-like cells [3, 4], that are an instrument in initiating mucosal immunity and a portal explored for the delivery of macromolecules and nanoparticles due to its high transcytotic capacity [5]. The 3D model was successfully established reproducing somewhat the native intestinal mucosa in which stromal cells play a pivotal role in the establishment of intestinal architecture. Intestinal myofibroblasts had a determinant role in the modulation of the surrounding matrix revealed by the existence of a fibronectin network that supported the epithelial cells. Although trigger M-like cells have revealing a challenge due to the inexistence of a distinct cell marker, the lack of microvilli was still the best cue to identify M-like cells. The transport of insulin through the 3D model showed to be modulated by the presence of mucus and the less tight character between the multiple epithelial cells that mimic the heterogeneous population along the crypt-villi intestinal axis.

Overall, the present 3D model has potential to be routinely adopted as a platform for high-throughput analysis of drug absorption and diagnostic of intestinal diseases.

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Face-to-Face: fetal- to adult-derived niches as 3-D *in vitro* culture systems for cardiac cells

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The paradigm of mammalian heart as a post-mitotic organ has been challenged following a set of reports showing the heart as capable of histo-functional restoration during fetal-neonatal life [1, 2]. Although different mechanisms have been implicated in heart remodeling/functional reestablishment, a full understanding is still far from reach. Owing to the well-recognized role of extracellular matrix (ECM) in heart morphogenesis and growth, we hypothesize that the native organ specific ECM provide developmental-stage dependent cues that may modulate cardiac cells' phenotype. This regulatory effect is likely to impact on tissue function in homeostasis as well as under injury. Thus, our work has been focusing on i) how the 3-D specific arrangement of fetal (E18) and adult cardiac tissues govern cardiac cell behavior and, on ii) dissecting further the presumptive underlying mechanisms.

Fetal and adult native cardiac ECM and ECM-associated factors were captured by a simple and quick original technology [3] enabling parallel decellularization of whole-fetal and adult-heart explants thereby ensuring reliable comparison between distinct stages cardiac-derived ECM. The acellular scaffolds produced display a complex network, preserving essential ECM components and evidencing intrinsic differences. When interacting with Lin-Sca-1$^+$ heart-derived progenitors [4] and neonatal cardiomyocytes in *in vitro* culture conditions a differential cellular response/dynamics was observed. Fetal-derived ECM revealed to be a more amenable niche for cardiac cells, presenting a higher number of cells per area, in contrast to the adult decellularized samples.

Future work will be directed to identify the mechanism(s) that control(s) this differential potential towards development of ECM-based therapies for improved heart repair/regeneration.

Overall, the present 3D model has potential to be routinely adopted as a platform for high-throughput analysis of drug absorption and diagnostic of intestinal diseases.

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CD8$^+$ T cells protect against *Neospora caninum* infection by producing interferon-γ

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It is well established that CD8$^+$ T cells play an important role in protective immunity against different protozoan infections. However, the role of these cells in the course of *Neospora caninum* infection is not completely elucidated. Here we show that CD8-deficient C57BL/6 mice infected with *N. caninum* presented a higher organ parasitic load than their wild-type counterparts. Moreover, adoptive transfer of CD8$^+$ T cells sorted from the spleen of *N. caninum*-infected immunosufficient C57BL/10 ScCr mice prolonged the survival of lethally susceptive IL-12 receptor-deficient C57BL/10 ScSn mice upon challenge with this parasite. Infection with *N. caninum* induced activation of and interferon-γ production by CD8$^+$ T cells, both in C57BL/6 and C57BL/10 mice, indicating that production of this cytokine mediates the host protective role of these cells in the course of neosporosis. In order to confirm this hypothesis, RAG-deficient mice were reconstituted with normal CD4$^+$ T cells plus CD8$^+$ T cells obtained from either IFN-γ-deficient or normal donors, and then infected with *N. caninum*. Mice that received IFN-γ-expressing CD8$^+$ T cells presented lower parasitic burden than counterparts that received IFN-γ-deficient CD8$^+$ T cells. As perforin-defective mice presented parasitic burdens similar to those detected in wild-type controls when infected with *N. caninum*, we conclude that production of IFN-γ rather than cytotoxicity is the main protective mechanism conferred by CD8$^+$ T cells in the course of neosporosis.

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Deregulation of iron metabolism during *Listeria monocytogenes* infection in mice is not dependent on hepcidin expression

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As iron is vital for all cells, host sequestration of iron (nutritional immunity) provides a significant barrier to bacterial infection. Consequently, anaemia may be a complication of chronic infectious diseases. Despite the extensive research over the last decade in iron metabolism and its deregulation during infection, the mechanisms by which anaemia occurs remain to be known. Hepcidin, which was firstly described as an antimicrobial peptide, is an iron-regulatory hormone, predominantly produced in hepatocytes, that has been considered a fundamental element orchestrating host response upon bacterial infection and thus in the development of the anaemia of inflammation and chronic infection. Our group showed that host iron overload, leads to increased susceptibility to *Mycobacterium avium*. On the other hand, *M. avium* infection leads to moderate anaemia, without any alteration on hepcidin levels. *Listeria monocytogenes* is a Gram-positive intracellular pathogen that has been used as a model to study innate and adaptive immunity. In this work, we aimed to test the alterations on iron metabolism in the host and its possible association with hepcidin expression in liver during intravenous infection with *L. monocytogenes*. C57bl6 male mice were infected with $1x10^4$ CFU *L. monocytogenes* or vehicle and sacrificed 24h, 48h, 72h and 96h later. Bacterial load was quantified, blood was collected to evaluate the erythron and serum iron parameters. Liver was harvested for the determination of gene expression by RT-PCR. From the analysed haematological parameters, we were able to observe a decrease on red blood cells (RBC) number and on mean corpuscular volume over the experiment. In addition, *L. monocytogenes* infection induced the reduction of serum-iron over the first 72h of infection, accompanied by the decrease of transferrin saturation (sTRF). In the last time point analysed (96h), serum-iron and sTRF values recovered to nearly basal levels. In terms of gene expression analysis, *L. monocytogenes* infection was shown to induce a transcriptional up-regulation of transferrin (48h and 72h post-infection) and interleukin-6 (IL6) 48h after infection, however with no significant impact on hepcidin levels. From the obtained data, although *L. monocytogenes* infection induces a deregulation on iron metabolism and an up-regulation of IL6 transcription no impact on hepcidin expression was observed. Thus, suggesting that a hepcidin-independent mechanism might be involved in the iron metabolism deregulation. This point will be investigated in future work.

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Unraveling the effect of *Listeria monocytogenes* infection on the mitotic phase of the host cell cycle

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Bacterial pathogens hijack host signal transduction pathways to interfere with cellular processes such as cytoskeleton assembly, intracellular trafficking and apoptosis control [1,2]. *Listeria monocytogenes* has been extensively used as a model organism for cellular microbiologists and helped to understand the mechanisms evolved by pathogens to subvert cellular functions and establish infection. We recently reported the capacity of *Listeria* to interfere with the host cell-cycle via the activation of DNA damage checkpoints during the S-phase [3], and we are now addressing the effects of the infection on mitosis.

The analysis by flow cytometry of nocodazole-synchronized cells showed that *Listeria* infection causes a delay in the exit from mitosis and entry in G1 phase of the host cell cycle. In line with this finding, time-lapse live imaging of *Listeria*-infected cells revealed that cells containing bacteria are able to divide, although mitosis takes longer to be accomplished. This extended mitosis phenotype was abolished by cell treatment with a spindle assembly checkpoint (SAC) inhibitor, suggesting that infected cells present a postponed resolution of SAC, an intrinsic mechanism that regulates the progression in mitosis.

The evaluation by fluorescence microscopy of *Listeria* infected cells revealed an increased number of misaligned chromosomes in cells undergoing mitosis, as compared to non-infected controls. Additionally, when analysing the position of the mitotic spindle, infected cells showed spindle misorientation.

Taken together, our results point to an effect of *Listeria* infection on the mitotic phase of the host cell cycle as a new strategy involved in the infectious process of this bacterial pathogen.

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Hes5 specifies cardiac fate in a time-dependent manner

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Cardiac specification occurs early during embryonic development, approximately at the same time as primitive hematopoietic progenitors emerge. These differentiation events rely on a specific orchestration of distinct signaling pathways in a spatial/temporal manner. Embryonic stem cells (ESCs) faithfully recapitulate embryonic development and provide valuable mechanistic information, particularly in early cell fate decisions. In this work we aimed to dissect how Notch pathway directs mesoderm into cardiac and hematopoietic derivatives in mouse ESCs. Thus, the expression of Notch target genes was analyzed upon Notch1 activation in mesodermal progenitors. Hes5 response suggested a direct and time-specific effect, *i.e.* rapid increase in the first 24 hours after which it diminished, and was further evaluated as a mediator of Notch1 activity. Downregulation of Hes5 reduced cardiac differentiation while favoring hematopoietic commitment, as indicated by differential expression of cardiac (Isl1, Tbx5, Myh6) and hematopoietic (Scl, Gata1) genes. In contrast, Hes5 overexpression led to increased transcription of cardiac progenitor genes while early hematopoietic markers were downregulated. Our data further demonstrate that Hes5 enhanced cardiac fate in a confined temporal window while its sustained activity compromised cardiac maturation. Furthermore, Isl1 and Scl were identified as putative effectors of Hes5 activity in cardiac specification, supporting a tight intercorrelation between hematopoietic and cardiac regulatory networks. These findings provide mechanistic insights into how Notch pathway controls cardiac specification and identify a novel role for Hes5 as a determinant player in the early cardiac molecular program.

Production and Characterization of Isoniazid-Loaded SLN for tuberculosis *treatment*

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Introduction: Pulmonary route is one of the most promising non-invasive ways for administer drugs. With development of nanotechnology, new systems have been developed in order to overcome conventional drug systems-related problems, such as low drug bioavailability, enzymatic and chemical drug degradation, and undesired toxicity [1]. Nonetheless, there is still lacking of chronic toxicity studies of nanocarriers when administered through pulmonary route, particularly in the case of antituberculosis-loaded nanoparticles for treating infections caused by *Mycobacterium tuberculosis* (MT) [2].
With this work we pretend to develop isoniazid-loaded Solid Lipid Nanoparticles (SLN) which will further functionalized with mannose, in order to attain a better targeting against alveolar macrophages, the main target of MT.
Methods: Different lipids to produce SLN (Witepsol E85, Stearic acid and Compritol 888 ATO). Isoniazid was dissolved in purified water in a different concentration range. 200 µL of aqueous solution of isoniazid was added to 200 mg of lipid, dissolved in 2 mL of dichloromethane containing 1 mg/mL of stearylamine. This mixture was sonicated during 30 seconds at 70% of amplitude, forming the primary emulsion, then transferred for PVA 1% aqueous solution following sonication at same conditions. The secondary emulsion was stirred during 3 hours for organic solvent evaporation.
SLN were characterized in terms of size, polydispersity index (Dynamic Light Scattering), zeta potential (Laser Doppler Anemometer) and association efficiency (HPLC-UV).
Results: Mean particle size of SLN composed by Compritol 888 ATO was higher (between 921 and 1105 nm) when compared with SLN constituted by Witepsol E85 and Stearic acid (438-609 nm and 615-629 nm, respectively). All SLN presented a polydispersity index higher than 0.30. Regarding zeta potential, it was positive for most of formulations (from +16 mV to +22 mV), with exception of SLN composed by Stearic acid, which was comprised between -14 mV and -19 mV. Moreover, the association efficiency of almost formulations ranged from 17% to 26%.
Conclusion: All produced formulations presented the suitable size for alveolar macrophages uptake [3]. Stearylamine confer positive charge to the SLN, however the negative charge of SLN constituted by stearic acid was due to stearylamine do not reach the external surface of the particle to confer positive charge [4] or its quantity is not enough to cause a positive potential zeta. Regarding association efficiency, the values achieved were low, requiring further optimization.

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The intermediate expression of CCRL1 reveals novel subpopulations of medullary thymic epithelial cells that emerge in the postnatal thymus.

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Cortical (cTECs) and medullary (mTECs) thymic epithelial cells provide inductive microenvironments for T-cell development and selection. The differentiation pathway of cTEC/mTEC lineages downstream of common bipotent progenitors at discrete stages of development remains unresolved. We have recently demonstrated that fetal TEC progenitors expressing cortical properties are able to generate mTECs. These reports support the idea that embryonic TEC precursors progress transitionally through the cortical lineage prior to commitment to the medullary pathway. Whether these transitional precursors exist in the postnatal thymus is unresolved.

Here, using IL-7/CCRL1 dual reporter mice that identify specialized TEC subsets, we show that the stepwise acquisition of CCRL1 is a late determinant of cTEC differentiation. Although cTECs expressing high CCRL1 levels (CCRL1^{hi}) develop normally in immunocompetent and *Rag2*$^{-/-}$ thymus, their differentiation is partially blocked in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ counterparts. These results unravel a novel checkpoint in cTEC maturation that is regulated by the crosstalk between TECs and immature thymocytes. Additionally, we identify new UEA^{+}mTEC subtypes expressing intermediate CCRL1 levels (CCRL1^{int}) that conspicuously emerge in the postnatal thymus and differentially express *Tnfrsf11a*, *Ccl21* and *Aire*. While rare in the fetal and in *Rag2*$^{-/-}$ thymus, CCRL1int mTECs are restored in *Rag2*$^{-/-}$-Marilyn TCR Tg mice, indicating that the appearance of postnatal-restricted mTECs is closely linked with T-cell selection.

Our findings suggest that alternative temporally restricted routes of new mTEC differentiation contribute to the establishment of the medullary niche in the postnatal thymus. As mTECs have a crucial role in T cell maturation and tolerance induction, our findings have implications in therapeutics aimed at modulating TEC niches in the adult thymus.

Potential of marine cyanobacterial isolates to produce bioactive compounds

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Continental Portugal has an extensive coastline facing the Atlantic Ocean, nevertheless not very much is known about the diversity of marine cyanobacteria, with only a few reports published. Cyanobacteria are important primary producers and many strains are N2-fixers, playing a key role in the marine environment. They are a valuable prolific source of bioactive compounds and some of these products are toxic to a wide array of organisms, including animals and humans. However, cyanobacteria can also produce secondary metabolites with promising therapeutic applications such as anticancer, antibiotic and anti-inflammatory activities. In a previous study, several cyanobacterial strains were isolated from intertidal zones on the Portuguese coast and characterized using a polyphasic approach [1]. In this current project, a preliminary screening indicated that these cyanobacteria did not have the genes encoding proteins involved in the production of conventional cyanotoxins. However, earlier it was shown that extracts of marine *Synechocystis* and *Synechococcus* were toxic to invertebrates, with crude extracts causing stronger effects than partially purified ones [2]. To further evaluate the potential of our isolates (marine cyanobacteria from a temperate region) to produce bioactive compounds, a PCR screening for the presence of genes encoding non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS), targeting the adenylation (A) and ketosynthase (KS) domains respectively, was performed using the abovementioned isolates. DNA fragments were obtained for more than 80% of the strains tested, and the results revealed that PKS are more ubiquitous than NRPS genes. Based on the sequences obtained, an *in silico* prediction of PKS and NRPS systems was also performed. Moreover, RT-PCR analyses revealed that these genes are transcribed under routine laboratory conditions in a set of selected strains. Furthermore, LC-MS analysis coupled with molecular networking (visual representations of mass-spectral data based on chemical similarity [3]) were used to search for metabolites of interest, as well as novel ones.

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An automated Matlab tool to quantify cell migration on micropatterned surfaces

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Cell migration is essential for a variety of physiological and biological processes, such as wound healing and tissue regeneration. Migration, as well as other cellular responses, is dependent on cell-surface interaction. Understanding how different topographical stimuli are converted into intracellular signaling responses that cause changes in cell functions is of major interest [1,2]. The main goal of this work was to evaluate the influence of isotropic and anisotropic topography on cells behavior, in terms of migration distance and velocity, using a newly developed Matlab tool to automate the process of tracking cells on patterned surfaces.

A combined methodology of sol-gel and soft-lithography was used to produce micropatterned SiO_2 thin films. Anisotropic and isotropic micropatterns were created on glass substrates using a stamping method. Spin-coating was used to create a flat SiO_2 surface. hBMSCs were seeded on top of the thin films and placed in a Spectral Confocal Microscope Leica TCS-SP5 AOBS. Time-lapse images were recorded every 5 min for a period of 18h. Cells were maintained at 37ºC with 5% CO_2 during the images acquisition. Afterwards, cells morphological analysis was carried out by SEM.

Time-lapse videos were analysed using the developed CellTracking tool which provides two different operation modes: automatic and manual. Considering the manual mode, the tracking process is performed by the user which tracks each individual cell throughout the time-lapse video duration. In the automatic mode, the software starts by identifying and removing the existing background pattern and then performs the cell tracking automatically, using a detection-association approach [3]. The background removal process is fundamental to perform cell tracking as the periodic background pattern would otherwise lead to erroneous cell detection and association. Given the cell locations in each frame of the time-lapse video, the software automatically computes the required parameters for cell mobility analysis. The results obtained considering manual or automatic cell tracking were similar for all the surfaces, which is an indicator of the good performance in the automatic cell tracking. It was clearly observed that cell mobility was dependent on the surface topography, since the migration velocity and distance were superior on the patterned surfaces. The anisotropic and isotropic surfaces showed a significantly higher migration and velocity when compared to the flat surface and with no differences between them. The use of an automated tool to track cells has the advantage of reducing the time spent on the manual tracking besides allowing the tracking of cells in surfaces with different topographies. CellTracking is an easy-to-use and reliable software for cell migration analysis.

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Leishmania extracellular vesicles: insights into their immunoregulatory role in the establishment of the infection

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The use of secretion pathways for effector molecule delivery by microorganisms is a trademark in pathogenesis. Recent emphasis has been given to the release of extracellular vesicles (EVs) by the parasite *Leishmania*, the etiological agent of leishmaniasis. The absence of vaccines and satisfactory treatments associated to this disease demand a better understanding of the infectious process. The evasion of the immune response is critical for infection establishment. Our hypothesis is that parasite exoproteome components could be implicated in these early evasion mechanisms by acting on immune cells. In this sense, the immunological potential of the promastigote exoproteome (EXO) and its associated components EVs and vesicle depleted exoproteome (VDE), has been tested, *in vitro* and *in vivo*, using Balb/c model. Subsequently, an air pouch model was used allowing the study of local cell recruitment. Mouse's backs were inflated and subsequently injected with EVs, VDE, EXO or parasites. At 6 and 24h after inoculation, animals were euthanized, the pouch exudate collected, and the presence of different cell populations studied by flow cytometry. For *in vitro* studies bone marrow-derived dendritic cells (DCs) and macrophages were used. The expression of several activation markers was studied by flow cytometry, after 24h of incubation with EVs, VDE, EXO or parasites. Moreover, the state of responsiveness of the cells was also evaluated by re-stimulation with LPS and Poly I:C. At the evaluated time points, the extracellular materials promoted differently immune cell recruitment. *In vitro*, all EVs, VDE and EXO, as well as the parasites, are able to diminish the response capacity of DCs and macrophages to TLR ligands suggesting that these components might play an immunomodulatory role in the establishment of the infection.

HOST INTERACTION AND RESPONSE | POSTER 19

IL1B signaling leads to increased cell survival of gastric carcinoma cells

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Polymorphisms in inflammation-related genes have been associated with risk to gastric carcinoma (GC). However, the biological mechanisms underlying these associations are still elusive. Our objective was to determine whether chronic inflammation-associated IL1B signaling, as seen in the context of *Helicobacter pylori* infection, can be linked to gastric carcinogenesis by modulating the behaviour of gastric epithelial cells.

The effect of IL1B was assessed by studying the expression and activation status of the IL1B-activated transcription factors C/EBPβ and CREB in GC cell lines. Interaction between CREB and C/EBPβ was explored through interference RNA, chromatin immunoprecipitation and chemical inhibition. CREB and C/EBPβ expression was analyzed in 66 samples of primary GC and in normal gastric mucosa. GC cells growth was analyzed *in vitro* by BrdU incorporation and *in vivo* employing a chicken embryo chorioallantoic membrane model.

We found that IL1B regulates the expression/activation status of both C/EBPβ and CREB in GC cells through an ERK1/2-dependent mechanism. Our results show that CREB is a direct transactivator of *CEBPB*, acting as an upstream effector in this regulatory mechanism. Furthermore, we found CREB to be over-expressed in 94% of GC samples and significantly associated with C/EBPβ expression ($P<0.05$). Finally, we demonstrate both *in vitro* and *in vivo* that CREB can mediate IL1B-induced GC cell proliferation.

Our results support the hypothesis that the effect of chronic inflammation on gastric carcinogenesis, as seen in the context of genetically susceptible individuals infected with *H. pylori*, includes modulation of signaling pathways that regulate survival mechanisms in epithelial cells.

HOST INTERACTION AND RESPONSE | POSTER 20

Exploring immunomodulatory biomaterials for more effective MSC recruitment

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Current regenerative strategies apply a combination of engineered scaffolds with stem cells and growth factors for repair/regeneration. The great majority of these strategies aim for spatial and temporal control of stem cell differentiation. For injury repair, specific progenitor cells are recruited and have to move coordinately, in a highly regulated manner. And indeed, effective bone repair can be stimulated by recruitment of Mesenchymal Stem/Stromal Cells (MSC) to the injury site, where these cells can differentiate into bone cells, exert immunoregulation or produce of soluble mediators. We have described that human NK cells and macrophages lead to increased MSC recruitment. Implanted biomaterials elicit an inflammatory response, whose delicate balance will determine the effectiveness of tissue repair/regeneration. Here, we aim to understand how to use different microenvironments to modulate the immune response and as a consequence regulate MSC recruitment.

For that, on one hand we started analysing the mechanism involved in NK cell-mediated MSC recruitment. It was found that chemokines are essential in this recruitment. Semi-quantitative antibody arrays and quantitative ELISA assays indicate that CCL5 (RANTES), GRO-gamma and IL-8 and not SDF-1 might be involved in this NK cell – MSC interplay. Simultaneously, we are analysing how 3D materials affect immune responses and consequently impact on MSC invasion. More specifically, we have developed a transwell system to evaluate how 3D scaffolds affect immune cell-mediated MSC invasion. Interestingly, human NK cells, monocyte or macrophage seeded in PLA and chitosan stimulated MSC recruitment at different extents.

Stromal Cell Derived Factor-1-delivery system: a new approach for the recruitment of Mesenchymal Stem Cells in degenerating intervertebral disc

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Introduction: Intervertebral disc (IVD) degeneration is the leading cause of low back pain and disability in the active population. Transplantation of mesenchymal stem cells (MSCs) in a hydrogel carrier can induce regenerative effects in degenerated IVDs. Moreover, it was found that degenerative discs release chemoattractants effective in MSC recruitment. Based on these findings, we hypothesized that an injectable hydrogel that can enhance the number of migrated MSCs in the IVD and provide a suitable matrix for their survival and differentiation would be ideal. The purpose of this study was to evaluate the potential of a thermoreversible hyaluronan-poly(N-isopropylacrylamide) (HAP) hydrogel as chemoattractant delivery system to recruit human MSCs in degenerative IVDs. For that purpose, we have incorporated Stromal Cell Derived Facto-1 (SDF-1), a well-known chemokine which is able to recruit hMSCs, into HAP hydrogel and assessed hMSCs migration in an *ex vivo* model of degenerating IVD. **Materials and Methods:** HAP was used as a platform for the delivery of SDF-1. Release studies were performed *in vitro* and in an *ex vivo* model system. To assess hMSCs migration, an *ex vivo* model was used. IVDs with vertebral endplate (VEP) were isolated from young bovine tails (n=10) and hMSCs were obtained from human bone marrow (n=5, 19/21/24/40/64 years old). A model of IVD injury through the VEP ("cavity") was adopted to mimic the process of disc degeneration. This cavity was filled with different formulations of the gel and gel with SDF-1, or remained empty and then sealed with cement. hMSCs previously labeled with PKH26 were seeded ($1x10^6$ cells/disc) on the top of VEP (opposite to the injury) of each disc and then incubated in during 48h. Cell migration was evaluated by fluorescence microscopy. **Results:** Migration of MSCs was observed in all the conditions tested, nonetheless significant enhancement of migration was observed in comparison to the controls, in the presence of SDF-1-hydrogels. The percentage of hMSCs found in the annulus fibrosus (AF) and nucleus pulposus (NP) was very similar, showing that cells are capable of degrade the matrix of both tissues. Cells from younger donors (n=3) showed a 3.7±0.5 fold increase in migration in comparison to those from older donors (n=2), suggesting that hMSCs isolated from younger donors are much more prone to migrate, especially in response to the injury created (the cavity), when compared to older ones. **Conclusions:** Overall, this study demonstrates that the HAP hydrogel is a suitable carrier for SDF-1 delivery in the IVD being able to recruit hMSCs towards the degenerating disc. With this strategy we expect to overcome the disadvantages associated with cell transplantation therapies, namely cell survival, taking advantage of hMSCs potential. In the future, we expect to investigate if cell recruitment could be a valuable approach for IVD regeneration *in vivo*, in particular in the enhancement of ECM synthesis. A successful approach to recruit hMSCs for IVD would represent a breakthrough innovation for treating IVD-associated conditions.

Sphingolipids as modulators of iNKT cell activation

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Invariant Natural Killer T (iNKT) cells are a subset of lipid specific T lymphocytes that play important roles in cancer, auto-immunity and infection. They are rapidly activated by antigenic lipids bound to CD1d molecules, producing large amounts of cytokines. We have previously identified iNKT cell alterations in Fabry disease, a lysosomal storage disease characterized by the accumulation of the sphingolipid globotriaosylceramide (Gb3). This lead us to hypothesize that Gb3 might be the responsible for the alterations found. Therefore, here we analysed Gb3 effect on iNKT cell activation.

Gb3 was not able to activate human iNKT cells, indicating that it does not behave as an iNKT cell antigen. However, when we added Gb3 together with the potent iNKT cell antigen αGalCer to human monocytes, we observed a decrease in iNKT cell activation. To exclude the possibility of an effect of CD1d downregulation, cytotoxicity or other CD1d-independent mechanism, we performed activation assays using an antigen presenting cell-free system, with plate-bound CD1d. With these experiments we clearly show that iNKT cell activation was reduced when Gb3 was added together with αGalCer. Importantly, this effect only applied to CD1d, since a plate bound assay with CD1b revealed no decrease in the stimulation of a CD1b-restricted T cell clone. Altogether, the aforementioned results strongly suggested that Gb3 might compete with αGalCer for binding to CD1d. This can be analysed by using the L363 antibody to measure the amount of mouse CD1d:αGalCer complexes at the surface of antigen presenting cells. We found that splenic dendritic cells cultured simultaneously with Gb3 and αGalCer displayed a reduction of such complexes. This reduction was accompanied by a decrease of CD1d expression at the cell surface.

To conclude, our results show that Gb3 acts on antigen presenting cells to reduce lipid antigen presentation in two different ways: by downregulating CD1d expression at the cell surface and by binding to CD1d preventing antigen loading. These results allow us to describe Gb3 as a modulator of iNKT cell activation.

Cellular characterization of pore-forming toxin induced bleb-like structures

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Many bacteria secrete pore-forming toxins (PFTs) during infection to promote disease. PFTs disrupt the plasma membrane (PM) integrity causing the influx of Ca^{2+} from the extracellular environment. This contributes to the stimulation of repair mechanisms such as PM blebbing, which allow isolation and recovery of membrane lesions. Blebbing is regulated by the actomyosin cytoskeleton, however the specific regulators of this process remain largely unknown. We found that challenge of host cells with different bacterial PFTs (Listeriolysin O (LLO) or Aerolysin O) induces Ca^{2+}-dependent blebbing followed by formation of bleb retraction structures at the cell surface. Characterization of these structures showed that they display a unique cytoskeletal organization and recruit different cytoskeletal components such as actin, non-muscle myosin-II (NMII) isoforms, Filamin and others. siRNA depletion studies showed that depletion of NMIIA inhibits the formation of these structures and renders cells strongly susceptible to intoxication. In contrast, NMIIB is not required for this process. In addition, the analysis of other cellular organelles showed that PFT intoxication causes a strong endoplasmic reticulum (ER) vacuolation and the recruitment of ER proteins to the bleb retraction structures. Interestingly, pre-treatment of host cells with Brefeldin A, an inhibitor of the ER-to-Golgi anterograde transport, reduces the number of bleb-like structures following PFT intoxication, suggesting an important role for the ER in the regulation of toxin-induced blebbing responses. Here we will characterize these distinct cytoskeletal structures and discuss the role of different cytoskeleton regulators and ER components during membrane blebbing and cell survival responses to PFTs.

KEY WORDS: Pore-forming toxins, non-muscle myosin IIA, Listeriolysin O, membrane blebbing;

Antimicrobial properties of membrane-active dodecapeptides derived from MSI-78

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Antimicrobial peptides (AMP) are a class of broad-spectrum antibiotics known by their ability to disrupt bacterial membranes and their low tendency to induce bacterial resistance, arising as excellent candidates to fight bacterial infections. In this study we aimed at designing short 12-mer AMP, derived from MSI-78 (22 residues), a highly effective and broad spectrum synthetic AMP, by truncating the peptide at the *N*- and/or *C*-termini while spanning the MSI-78 sequence with 1 amino acid (aa) shifts [1,2]. These designed peptides were evaluated regarding antimicrobial activity against selected gram-positive *Staphylococcus* strains and the gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*).

In general most of the short 12-mer peptides tested showed a reduction in antimicrobial activity, an effect that was more pronounced for gram-positive *Staphylococcus* strains. However, the 12-mer peptide CEM1 (GIGKFLKKAKKF) arose as a good candidate to fight *P. aeruginosa* infections, as it displays antimicrobial activity against this strain and selectivity, with negligible toxicity to mammalian cells even at high concentrations. Interestingly, CEM1 and a highly similar peptide differing by only one aa-shift (CEM2: IGKFLKKAKKFG), showed a remarkably contrasting AMP activity. These two peptides were chosen for a more detailed study regarding their mechanism of action, using several biophysical assays and simple membrane models that mimic the mammalian and bacterial lipid composition.

We found a direct correlation between peptide helicity and antimicrobial activity and propose a mechanism of action that supports an antibacterial action based on bacterial membrane lysis and consequent bacterial cell death.

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Neuropeptide Y Y_1 receptor in Bone remodeling and Regeneration: a key player *versus* a therapeutic target

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Introduction: Over the past decade great emphasis has been given to the pronounced anabolic effects in bone metabolism induced by the global or osteoblast-specific disruption of the Neuropeptide Y Y_1 receptor (Y_1R) [1,2]. These data suggested that an anti-Y_1R drug therapy might have clinical applications for the prevention/recovery of bone loss, such as osteoporosis. Therefore, we investigated the effects of a highly selective non-peptidic Y_1R antagonist (BIBO3304) on bone metabolism. In addition, given the high fracture incidence in the target population, to complete this study we have also evaluated the role of Y_1R in the control of bone fracture healing.

Materials and Methods: Wild-type (WT) mice were treated daily with increasing doses (0, 0.5 and 5 µmol/mouse/day) of BIBO3304 for 8 weeks. The drug was incorporated into a jelly and administered orally to mice in a daily basis. Bone remodeling alterations were analyzed by densitometry, µCT and histomorphometric analysis on isolated femurs. Closed tibial fractures were also generated in WT, germline ($Y_1R^{-/-}$) and osteoblastic-specific Y_1R knockout mice. Fracture repair was monitored by weekly radiography and radiographs were used to grade fractures for union. At the experimental endpoint of 3-weeks and 6-weeks post-fracture, structural alterations were evaluated by µCT analysis and morphological changes were also quantified on histological images of fracture calluses.

Results and Discussion: 8-weeks of 5 µmol BIBO3304-treatment induced a significant enhancement on whole body bone mineral content when compared to vehicle-treated mice, which resulted in greater femoral cancellous bone volume, trabecular number and thickness, and in association with an increase in mineral apposition rate. These results are consistent with data previously described for the germline $Y_1R^{-/-}$ mice [1].

Moreover, in what regards fracture healing, the $Y_1R^{-/-}$ mice exhibited a smaller callus, when compared to WT fracture calluses. In fact, at 3-weeks post-fracture 75% of fractures were still not bridged, revealing a delay in fracture healing in $Y_1R^{-/-}$ mice, when compared to the 89% of completely bridged in WT mice. Nevertheless at 6-weeks post-fracture, all $Y_1R^{-/-}$ and WT fracture calluses were completely bridged, suggesting that a delay in the early stage of bone fracture bridging had occurred in $Y_1R^{-/-}$ mice. These results were further corroborated by µCT quantification, where the analysis of fracture calluses scanning images revealed a significant decrease on bone/tissue callus volume, percentage of mineralized callus and on callus strength in $Y_1R^{-/-}$ mice, possibly associated with a delay in soft callus remodeling and cartilage removal. Interestingly, this delay in bone repair was not related directly to Y_1Rs expressed by mature osteoblasts, given the fact that no structural or morphological differences on callus formation were observed in osteoblastic-specific $Y1R^{-/-}$ mice.

Conclusions: This work enabled us to conclude that although the use of Y_1R antagonists might be an useful tool to stimulate bone formation, the importance of Y_1R on the early stages of fracture healing must also be taking into consideration. Specifically when envisioning a local therapeutic approach, such as the design of an anti- Y_1R delivery system to promote bone repair in large defects.

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Modulation of the Inflammatory Response to Chitosan Scaffolds through M2 Macrophage Polarization Using Pro-resolution Mediators

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Tissue engineering and regenerative medicine have created a demand for biomaterials with specific functions such as the ability to modify the host immune response. The objective of this study was to evaluate the effect of two different pro-resolution lipid mediators, lipoxin A4 (LxA4) and resolvin D1 (RvD1), in the modulation of the inflammatory response to biomaterials through M2 macrophage polarization. This was investigated *in vivo* using a mouse air-pouch model of inflammation. Our results demonstrate that both LxA4 and RvD1 are able to shift the macrophage response to the implanted Ch scaffolds to an M2 reparative response. The injection of these pro-resolution mediators caused a decrease of the inflammatory cells recruited to the implant site together with higher numbers of F4/80+/CD206+ cells (M2 macrophages) and lower numbers of F4/80+/CCR7+ cells (M1 macrophages); it also induced a general decrease in several pro-inflammatory cytokines; and caused a significant decrease in the thickness and area of the fibrous capsule formed around the implanted scaffolds. In conclusion, the use of either LxA4 and RvD1 allowed the *in vivo* control of macrophage phenotypic profile and thus may play a significant role in regenerative medicine applications, namely through modulation of inflammatory response.

Can magnesium ions induce macrophage polarization?

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Owing to the unique properties of metallic ions, new standards of tissue healing may be achieved, namely through strategies to modulate immune response towards tissue regeneration [1]. Macrophages are key players in foreign body reaction against implantable biomaterials and depending on the microenvironment, macrophages may differentiate in cytotoxic (M1) or pro-regenerative phenotype (M2). Magnesium-based biodegradable metallic alloys are being developed for orthopedic applications but the influence of the released Mg^{2+} ions on immune response and subsequent tissue regeneration is quite unknown. Thus, this work aims at determining potentially therapeutic concentrations of Mg^{2+} ions that can influence the behavior of macrophages towards regenerative phenotype.

Monocytes were isolated from human buffy coats as previously performed by our team [2]. After isolation, monocytes (5 donors) were continuously exposed to concentrations of $MgCl_2$ up to 100 mM during 14 days in 2 different cell culture media (RPMI and DMEM). Cellular metabolic activity was evaluated at day 1,3,7 and 14 by Alamar Blue assay. Cell apoptosis was assessed by flow cytometry, using annexin V/PI staining. Using non-toxic concentrations, cell morphology was evaluated by fluorescence microscopy, upon cytoskeleton (F-actin) and nuclei (DAPI) staining. The differentiation of macrophages in M1 or M2 phenotypes was evaluated through quantification of cytokines (IL-6, IL-10 and IL-12/IL-23) and growth factors (TGF-B1) in supernatants by ELISA.

Concentrations of above 10 mM of $MgCl_2$ were found to be toxic. Increased metabolic activity was observed in macrophages exposed to concentrations of 0.5 mM and 5 mM of $MgCl_2$. Macrophage stretched morphology and a slight increase of apoptotic cells (from 5% to 15%) were also associated with presence of 5 mM of $MgCl_2$. Interestingly, increased production of TGF-B1, an important factor involved in bone repair/regeneration and secreted by M2 macrophages was registered was found in macrophages exposed to concentrations of 0.5 mM and 5 mM of $MgCl_2$. No differences in comparison with the control (0 mM $MgCl_2$) were detected for IL-10 or cytokines overexpressed by M1 macrophages (IL-6 and IL-12/IL-23).

These preliminary results pointed that Mg^{2+} ions seem to influence macrophage differentiation towards regenerative phenotype M2. In future work, full characterization of macrophage phenotype through flow cytometry will be carried out to assess the expression of other phenotypic markers of M2 population.

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TLR2-Induced IL-10 Production Impairs Neutrophil Recruitment to Infected Tissues during Neonatal Bacterial Sepsis

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Sepsis is the third most common cause of neonatal death, with Group B Streptococcus (GBS) being the leading bacterial agent. The pathogenesis of neonatal septicemia is still unsolved. We described previously that host susceptibility to GBS infection is due to early IL-10 production. In this study, we investigated whether triggering TLR2 to produce IL-10 is a risk factor for neonatal bacterial sepsis. We observed that, in contrast to wild-type (WT) pups, neonatal TLR2-deficient mice were resistant to GBS- induced sepsis. Moreover, if IL-10 signaling were blocked in WT mice, they also were resistant to sepsis. This increased survival rate was due to an efficient recruitment of neutrophils to infected tissues that leads to bacterial clearance, thus preventing the development of sepsis. To confirm that IL-10 produced through TLR2 activation prevents neutrophil recruitment, WT pups were treated with the TLR2 agonist Pam3CSK4 prior to nebulization with the neutrophil chemotactic agent LTB4. Neutrophil recruitment into the neonatal lungs was inhibited in pups treated with Pam3CSK4. However, the migration was restored in Pam3CSK4- treated pups when IL-10 signaling was blocked (either by anti–IL-10R mAb treatment or by using IL-10–deficient mice). Our findings highlight that TLR2-induced IL-10 production is a key event in neonatal susceptibility to bacterial sepsis.

The impact of hLF1-11 antimicrobial peptide immobilization on its antimicrobial activity

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Introduction: Antimicrobial peptides are recognized as a promising new class of antimicrobial agentes. They are known for their broad spectrum of activity, even against antibiotic-resistant bacteria, and their low tendency to induce resistance [1]. hLF1-11 is a AMP derived from human Lactoferrin with such characteristics, whose clinical value was tested as the antimicrobial agent in a calcium-phosphate cement delivery system on an implant application [2]. Although, some activity was found, the burst release, and rapid clearance of the peptide from the local, suggested that a different administration strategy should be pursued. Covalent immobilization of AMP has been suggested as a way to locally enhance the activity period of time, while avoiding some of the peptide free form issues: fast degradation, peptide aggregation and/or peptide toxicity when used at high titers [3]. The aim of this study was to evaluate if hLF1-11 tethering could solve its application problems. To this end, two different immobilizations were tested: direct immobilization onto a polymer and spacer-derived immobilization [4].

Experimental Methods: Chitosan solution was spinned onto Gold substrates to obtain ultrathin films, that were then further functionalized through carbodiimide chemistry with N-acetyl cysteine or a spacer ($SHCH_2$-$[OCH_2CH_2]_7$-OCH_2CH_2COOH), in order to assess direct vs flexible immobilization of the peptide. hLF1-11 was then immobilized onto these functionalized surfaces establishing a disulfide bridge between the free –SH of modified chitosan and the –SH of the hLF1-11 cysteine, under oxidative conditions (DMSO). Full surface characterization was obtained through X-ray Photoelectron Spectroscopy, Infrared reflection absorption spectroscopy, ellipsometry, water contact angle, Atomic Force Microscopy and surface peptide quantification. The antimicrobial activity of the hLF1-11 was firstly assessed in its soluble form through a Minimal Inhibitory Concentration assay with Methicillin-resistant *Staphylococcus aureus* ATCC 33591. Then the modified surfaces were tested with 10^7 CFU for 4h at 37ºC. At the end of this incubation period, different sample batchs undertook different assays: the supernantants were serially diluted and plated, for total adhered bacteria counts the surfaces were stainned with VECTASHIELD® Mounting Medium with DAPI, and for viable adhered bacteria counts, samples were sonicated, serially diluted and plated.

Conclusions: hLF1–11 covalent immobilization was successfully performed using specific orientation through its C-terminal cysteine, with N-acetylcysteine and spacer in similar amounts. Chitosan thin films by themselves decreased bacterial adhesion. The functionalization with hLF1–11 increased significantly bacterial adhesion to chitosan films, particularly when the peptide was covalently coupled without a spacer. However, when a spacer is used, hLF1–11 maintained part of its antimicrobial activity.

KEYWORDS: Antimicrobial Peptide; Surface immobilization; Bacterial adhesion

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L-rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to antimicrobial peptides by delaying interaction with the bacterial membrane

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Listeria monocytogenes (*Lm*) is a Gram-positive pathogen responsible for human listeriosis, an infectious disease that targets immunocompromised hosts, pregnant women and fetuses with higher incidence and morbidity. Our previous study of the *in vivo* transcriptome of *Lm* revealed that the *rmlACBD* cluster is significantly up-regulated during mouse infection[1]. This cluster encodes the biosynthetic pathway of L-rhamnose, a sugar that decorates *Lm* cell envelope glycopolymers known as wall teichoic acids (WTAs)[2]. In this work, we investigated the contribution of this WTA decoration to *Lm* virulence.

We showed by HPLC analysis that the presence of L-rhamnose in *Lm* WTAs is not only dependent on the expression of *rmlACBD* but also of the upstream gene *rmlT*, which encodes a putative rhamnosyltransferase. We demonstrated that WTA substitution with L-rhamnose is required for full virulence of *Lm* in mice. In addition, absence of WTA L-rhamnosylation resulted in decreased *in vitro* and *in vivo* resistance to cationic antimicrobial peptides (CAMPs), a family of amphipathic molecules that promote bacterial death *via* a disruptive insertion into the plasma membrane[3]. Using fluorescence techniques and electron microscopy, we showed that the increased CAMP susceptibility levels of *Lm* strains lacking L-rhamnose-decorated WTAs appear to be related to a significantly faster rate of cell wall crossing and plasma membrane destabilization by CAMPs. This observation is supported by evidence showing that the presence of L-rhamnosylated WTAs hinders the progression of CAMP molecules through the *Lm* cell wall and thus delays their harmful interaction with the plasma membrane.

This work revealed a role for L-rhamnose-decorated WTAs in *Lm* virulence and antimicrobial resistance, demonstrating for the first time the contribution of WTA glycosylation to important biological processes in this pathogen.

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Improving *Synechocystis* sp. PCC 6803 towards a more functional and robust photoautotrophic chassis

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Cyanobacteria are appealing organisms to be used in biotechnological applications as "low cost" cell factories due to their simple nutritional requirements and high metabolic plasticity. The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) is the best studied strain since its genome was the first to be sequenced amongst photosynthetic organisms, and plenty of genomic, transcriptomic and proteomic data are available. These data allowed the construction of genome-scale metabolic models (e.g. *iSyn811*), which are important tools to predict fluxes and responses to changes when engineering this organism. Moreover, *Synechocystis* is naturally transformable and the protocols for its genetic manipulation are well established. The availability of well characterized tools for the construction and implementation of genetic circuits is an important aspect to use this cyanobacterium as a chassis for Synthetic Biology applications. In this sense, and to improve *Synechocystis* functionality, we have identified and characterized several chromosomal loci to be used as neutral sites for the implementation of synthetic devices/gene circuits, and the integrative vectors to use these sites are already available. In addition, several genetic parts for the construction of synthetic devices were designed and are under characterization, namely constitutive and regulated promoters, RNA thermoswitches and enhanced reporters. Given the autotrophic nature of cyanobacteria, these organisms can be cultivated in outdoor bioreactors using sunlight as energy source. Moreover, the use of seawater as growth medium would be advantageous. Nonetheless, in this conditions they become exposed to environmental factors such as temperature and irradiation fluctuations. Therefore, the tolerance limits of the mesophilic and moderately halotolerant *Synechocystis* to temperature and salt concentrations has been assessed and several strategies to improve the chassis robustness were designed and are being implemented.

A molecular portray at the onset of the cardiac program: Dissecting the role of Notch in the determination of distinct cardiac lineages/fields

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Heart formation is a complex morphogenetic process resulting from the contribution of distinct signaling pathways that crosstalk in a temporal and context-specific manner. Differentiating mouse embryonic stem cells (mESCs) recapitulate *in vitro* the molecular events from pre-gastrulation up to cardiomyocyte formation contributing with mechanistic insights into early lineage specification. Likewise, the generation of different subsets of cardiac progenitors *in vitro* seems to mimic the emergence of cardiac lineages/fields which build the embryonic heart. First (FHF) and second (SHF) heart fields have been discriminated by expression of specific transcription factors, e.g. Tbx5 *vs.* Isl1, respectively, and differential temporal contribution to distinct cardiac territories. Importantly, the recent finding that HCN4, a voltage-gated ion channel, identifies FHF progenitors, in the embryo and human ESCs, enables the isolation of such progenitors based on the expression of this surface protein. Observations from our laboratory and others demonstrated a role for Notch signaling in cardiac- over hematopoietic- specification from primitive mesodermal progenitors. Following these findings, the herein work aims at dissecting the role of the Notch pathway in the determination of distinct cardiac lineages/fields using the mESCs *in vitro* model-system. A temporal mapping for the emergence of FHF and SHF over the time course of mESCs differentiation based on expression of the typical markers e.g. Tbx5, Isl1, Hcn4 was performed. Our preliminary results on the kinetics of development of the distinct cardiac-lineages will be shown. Future work contemplates the identification of the role different Notch effectors might play in each cardiac lineage/fields specification.

Public Awareness for Hereditary Hemochromatosis - The Portuguese Case Study

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Hereditary Hemochromatosis is one of the most common adult-onset genetic disorders in European-derived populations but underdiagnosed and undertreated, resulting from the general lack of knowledge about the disease. Similarly to what happens with other genetic diseases, HH requires adequate public awareness and effective appropriation of scientific knowledge about transmission, symptoms, diagnosis and treatment.

The Portuguese Association of Hemochromatosis (APH) has long endeavored to promote a social network for awareness of the disease. However, despite the role of Patient Organizations in targeting multiple audiences being clearly recognized, APH has had difficulty leveraging its forms of action.

The case study presented here demonstrates that the inclusion of a knowledge broker in civil poorly consolidated society organizations may be necessary for the capacity building of these organizations. The project, initiated within the Biosense platform, subsequently became independent through a PhD grant (BD-FCT-SFRH/BD/91672/2012), it is based on a qualitative/intensive methodology and on collaborative action research.

An initial and informal approach was made by APH to the Biosense platform, which enabled the identification and integration of a knowledge broker in the process, a PhD student in the Doctoral Program in Teaching and Dissemination of Science.

After designing the project concept, the initial phase of the work consisted in identifying all the stakeholders involved in the process of prevention, diagnosis and treatment of HH, including government health officials, researchers, health professionals, patients, carriers of the disease and the general public, from research, health, clinical and civil society organizations, namely the Institute for Molecular and Cell Biology (IBMC), the CHP-Hospital of Santo António, the Center for Predictive and Preventive Medicine (CGPP), the Portuguese Association of Hemochromatosis (APH) and the European Federation of Patients with Haemochromatosis (EFAPH).

The second phase has been planned and implemented collaboratively. A range HH outreach strategies (e.g. Symposium for General Practitioners and HH European Awareness Week ("Hemo… Quê?" School Contest; "António Leitão" Walk)) are being systematized and could be used as a reference for the preparation of new HH awareness projects in other countries. Within this project, too, collaboratively elaborated materials about HH and appropriate for different audiences have been produced (e.g. video and booklet). These actions constitute excellent means of HH dissemination and should contribute to the sustainability of the Patient Association.

Treatment of a degenerative/pro-inflammatory intervertebral disc organ culture with Chitosan/Poly-ɣ-glutamic acid nanoparticles carrying an anti-inflammatory drug

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Introduction: Low back pain related with intervertebral disc (IVD) degeneration is a major cause of lack to work in industrialized countries. Inflammation has been correlated with disc degeneration, although its role in discogenic pain remains controversial.[1] Here we purpose first to establish a pro-inflammatory disc organ culture model. Our goal is to evaluate a new anti-inflammatory therapy based on Chitosan (Ch)/Poly-(γ-glutamic acid) (γ-PGA) nanoparticles (NPs) with an anti-inflammatory drug (Diclofenac, Df) incorporated, previously developed by us.[2] Previous studies have shown that this delivery system was efficient in reducing macrophage activation *in vitro*.

Experimental Methods: Bovine caudal disc punches cultures (DMEM with 5% FBS and 5 mM glucose, 400 mOsm and 6% CO2 during 5 days) were needle-punctured and stimulated with: Lipopolysaccharide (LPS) 10 µg/mL, or with Interleukin-1β (IL-1β), 10 and 100 ng/mL, for 48h. The effect of the pro-inflammatory stimulus was evaluated by gene expression of pro-inflammatory cytokines (IL-6, IL-8), metaloproteases (MMPs, MMP1 and MMP3), and extracellular matrix (ECM) proteins (collagen type II (Coll II), Aggrecan). Cell viability was analyzed by LIVE/DEAD assay while metabolic profile of the cultures was traced by Glucose/Lactic acid monitoring.

As an anti-inflammatory therapy, Ch/Df/γ-PGA NPs prepared by co-acervation method[2], were added to disc cultures 3h after pro-inflammatory stimulus. Untreated samples were used as controls. The effect of the anti-inflammatory therapy was evaluated by gene expression and by Prostaglandin E_2 (PGE_2) production.

Results and discussion: To establish a pro-inflammatory *ex vivo* model of IVD, LPS and IL-1β treatments were compared. IL-1β-treated discs showed a statistically significant up-regulation of the pro-inflammatory cytokines (IL-6 and IL-8), MMPs expression (MMP1 and MMP3), while ECM proteins (Coll II and Aggrecan) were significantly down-regulated (Fig.1). For all the conditions tested, cells remain viable and presented similar metabolic activity. IL-1β stimulation was selected as the most adequate approach to study anti-inflammatory therapies for IVD.

Regarding the effect of NPs as Df-delivery system in the IVD organ cultures, results showed significant down-regulation of IL-6 and MMP1, and up-regulation of Coll II and Aggrecan, when compared to IL-1β stimulated groups, suggesting that this treatment not only reduces inflammation, but also can improve matrix proteins production.

PGE_2 levels were reduced in the presence of Df-NPs for

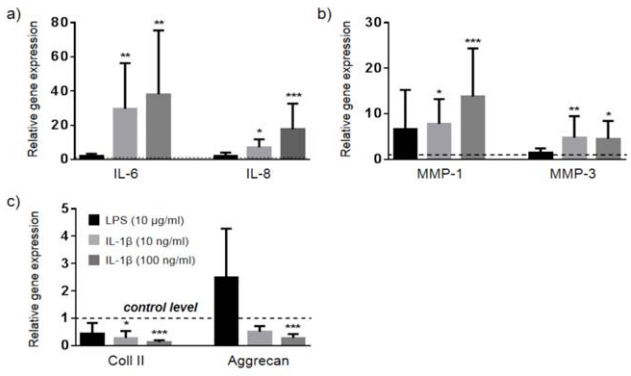

FIGURE 1: Quantitative analysis of pro-inflammatory markers of IVD organ cultures with pro-inflammatory stimulus (LPS and IL-1β). mRNA expression of IL-6 and IL-8 (a), MMP-1 and MMP-3 (b) and Coll II and Aggrecan (c). Levels of mRNA were normalized to GAPDH and to the control group. Results are shown as Mean ± StDev (n=4-10). * = p<0.05; ** = p<0.025; *** = p<0.001.

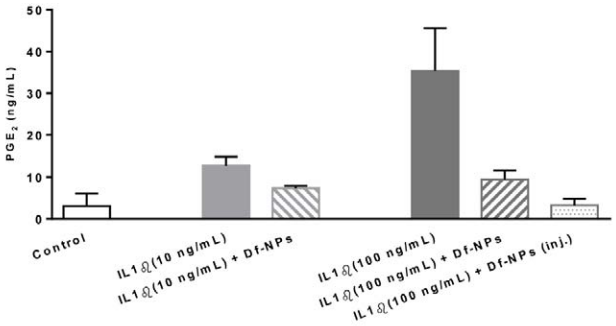

FIGURE 2: PGE_2 concentration detected at day 8, in culture medium supernatant, in the presence and absence of pro-inflammatory stimulus (IL1β) and anti-inflammatory treatment with Ch/Df/PGA NPs. Results are presented as Mean ± StDev (n=2-4).

both IL-1β concentrations, indicating that, in this range of concentrations, the effect of released Df is not IL-1β concentration-dependent. We have compared the Df-NPs injection in IVD with Df-NPs in IVD culture medium. The results indicate that injection group induces a better response (Fig.2).

Conclusion: An *ex vivo* model of degeneration/inflammation in IVD was here established, with increased levels of pro-inflammatory cytokines and MMPs and reduced ECM protein levels. This approach is suitable for *in vitro* testing of regenerative or anti-inflammatory strategies of disc degeneration. Ch/Df/γ-PGA NPs revealed to be a promisor anti-inflammatory therapy to IVD.

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Novel functions for the cytosolic peroxiredoxins of *Leishmania infantum*

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The family of Trypanosomatidae protozoa comprises human parasites with clinical relevance, including *Leishmania* spp., *Trypansoma cruzi* and *T. brucei*, causative agents of leishmaniasis, Chagas' disease and sleeping sickness, respectively. In these parasites, enzymes of the peroxiredoxin (PRX) family are well-known virulence factors. The critical function of these molecules for parasite survival is consensually ascribed to their capacity to detoxify hydroperoxides, including those produced during the oxidative assault mounted by the immune system of mammalian hosts. However, recent data from our lab have shown that the essential role of trypanosomatid PRXs may be unrelated to mere antioxidant protection. Based on this premise, we are currently exploring novel functions for these enzymes in *Leishmania infantum*. Specifically, we are investigating the involvement of cytosolic PRXs in H_2O_2-mediated signaling and how this contributes to parasite infectivity. Our work employs molecular, cell biology and biochemistry approaches.

Using the photoswitchable protein Dendra2 to trace human MSC in 3D Microenvironments

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One of the most promising cell types for cell-based therapies - from bone regeneration to treatment of autoimmune diseases - are Mesenchymal Stem/Stromal Cells (MSC), due to their capacity to migrate effectively to damaged tissues, to differentiate in different lineages, to their immunomodulatory and to their paracrine properties. Therefore, their use in tissue engineering applications holds great promise for tissue regeneration. Unexpectedly, the single and collective dynamical behavior of these cells in 3D microenvironments remains poorly characterized, due to the lack of tools to trace them in 3D. Here, we report on a simple and reliable bioimaging method to longitudinally follow MSC in engineered 3D matrices. This method is based on using MSC transfected to express the green-to-red photoswitchable protein Dendra2, and use it as a tool to stably highlight and follow the same subpopulation of cells for long periods of time. We show that Dendra2+ human bone marrow MSC maintain their multilineage differentiation capacity, surface markers phenotype and oriented migration capacity. Moreover, photoconversion was performed in labeled MSC without affecting cells survival, migration velocity and direction. Most importantly, photo-marked cells were accurately tracked for more that 7 days with no mistaken identity issues, while also allowing analysis of the cells morphology. Using this technique, it was found that MSC migrate more in Matrigel 1:1, 1% alginate-RGD or in chitosan loosen matrices when compared with tighter Matrigel 1:12 or 2% alginate-RGD counterpart. Thus, the established bioimaging tool provides a promising platform to characterize MSC dynamical behavior in bioengineered matrices that could be an allied for the development of novel biomaterials that aspire to improve stem cell recruitment and migration.

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Characterization of Y1 Receptor-Antagonist Binding in Bone Marrow Cells

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G-protein coupled receptor (GPCR) desensitization is a physiological process characterized by the loss of functional receptors at the cell surface. It constitutes an important step for the maintenance of cellular homeostasis. For a long time, it was believed that receptor desensitization would only occur as a consequence of agonist stimulation and required receptor activation. However, further studies showed GPCR internalization upon antagonist stimulation [1]. As antagonist-mediated receptor downregulation may have important therapeutic implications, the better understandingGPCR-antagonistinternalizationmechanisms are of upmost relevance.

Recently, Y1 receptor (Y1R) has arisen as a potential regulator in the local control of bone turnover [2]. Despite the various antagonists synthesized over the last decade there are very few studies on the antagonist regulation of Y1R [3] and no data concerning their mechanisms in bone marrow (BM) cells.

BIBP3226 is a potent Y1R selective antagonist that was successfully used in *in vitro* studies showing a positive impact in the benefit of bone turnover, thus providing good perspectives for its use as a pharmacological tool for bone regeneration [4].

In this study we use Image flow cytometry and confocal microscopy approaches to elucidate the mechanisms underlying the local effect of Y1R during bone repair and to characterize Y1R-BIBP3226 interaction within the BM cell population.

The obtained results allow a preliminary understanding of the mechanisms underlying the local effect of Y1R during bone repair and attained the characterization of Y1R-BIBP3226 interaction within BM cell population.

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Exploring the glycan-adhesin specific interaction to treat *Helicobacter pylori* gastric infection

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Introduction: *Helicobacter pylori* (*H. pylori*) is a bacterium that infects half of the world population [1] and its persistent infection is associated with development of gastric carcinoma. Standard antibiotic treatment is fallible in ~20% of the cases [2], therefore alternative strategies urge. H. pylori blood group antigen binding adhesin (BabA) recognizes Lewis b (Leb) and H-type 1 [3] and sialic acid binding adhesin (SabA) bind sialyl-Lewis a (sLea) and sialyl-Lewis x (sLex) [4] expressed in gastric mucosa. This work aims to develop a biomaterial which can be orally administrated, bind *H. pylori* in the stomach, removing them through the gastrointestinal tract. We have previously shown that 170µm genipin crosslinked chitosan microspheres are stable in acidic pH, have mucoadhesive properties, are not cytotoxic, are retained in the stomach of C56BL/6 mice for at least 2h and bind unspecifically different strains of *H. pylori* [5,6]. We herein evaluate the capacity of glycan-decorated chitosan microspheres (GlyR-Mic) to bind *H. pylori* through specific glycan-adhesin interactions and compete for bacterial adhesion with mice and human gastric mucosa.

Materials & Methods: Chitosan microspheres (Ch Mic; d=170µm) were produced and GlyR, namely Leb and sLex, immobilized by "click chemistry" [7]. Adhesion of FITC-H. pylori strains expressing different functional adhesins (BabA and/or SabA) to GlyR Mic was visualized by confocal microscopy. Competition assays for *H. pylori* adhesion between Mic and gastric mucosa were performed using two *in vitro* models and one *ex vivo* model, all expressing Leb. Regarding *in vitro* studies, Mic were added before (prevention) or after (removal) the FITC-*H. pylori* incubation with sections of paraffin embedded gastric mucosa from human and mice. Bacterial adhesion levels were quantified using ImageJ software. The *ex vivo* model was designed to evaluate the ability of Mic to remove/prevent [35S]-radiolabeled *H. pylori* adhesion to fresh mice gastric mucosa, quantified using a luminescence counter.

Results/Discussion: Glycan-adhesin specific *H. pylori* adhesion was confirmed since BabA+/SabA- *H. pylori* strain adhered to Leb-Mic but not to sLex-Mic. Unlike sLex-Mic, Leb-Mic were able to compete with Leb expressed in all gastric mucosa models tested using a BabA+ *H. pylori* strain. Moreover, Leb-Mic presented higher bacterial removal and prevention rates than Ch-Mic. Regarding the *in vitro* model of mice and human gastric mucosa, statistically significant differences were observed for bacteria removal rates of ~60% and ~43%, and prevention rates of ~32% and ~35% using Leb-Mic, respectively. The *ex-vivo* model using fresh mice gastric stomach also suggested Leb-Mic ability to both remove (~65%) and prevent (~78%) *H. pylori* adhesion from/to these gastric sections.

Conclusion: *H. pylori* adhesion to GlyR-Mic is ligand specific and efficiency studies revealed their potential as alternative or complementary treatment to *H. pylori* gastric infection.

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Ribose 5-phosphate isomerase B knockdown compromises *Trypanosoma brucei* bloodstream form infectivity

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Ribose 5-phosphate isomerase (Rpi) is involved in the non-oxidative branch of the pentose phosphate pathway and catalyses the inter-conversion of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P). There are two non homologous forms, the A and the B. The presence of type B in trypanosomatids, and its absence in humans, points RpiB as a potential drug target. This study presents a functional characterization of *T. brucei* ribose 5-phosphate isomerase B (*Tb*RpiB). *In vitro* biochemical studies confirmed *Tb*RpiB isomerase activity, as it can use both R5P and Ru5P as substrates. *Tb*RpiB knockdown by RNAi affected *in vitro* growth of bloodstream forms, but more importantly *in vivo* parasites infectivity since mice infected with induced RNAi clones exhibited lower parasitemia and a prolonged survival in comparison to mice infected with control parasites. Furthermore, *in vitro* and *in vivo* phenotype was reverted when an ectopic copy of *Trypanosoma cruzi* ribose 5-phospahate isomerase was introduced. These results suggest *Tb*RpiB as a promising drug target for African sleeping sickness, since interfering with this protein represents a way to control *in vivo* parasite growth and infectivity.

Characterization of the neonatal innate immune response to *Escherichia coli* infection

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Escherichia coli is the most common cause of Gram-negative neonatal sepsis and meningitis, with a case fatality rate of 15-40% and severe neurological defects in many of the survivors. The usual treatment is based on the use of antibiotic which leads to a great health concern due to the emergence of resistant strains. A comprehensive view of the neonatal immune system responding to bacterial infection and of the mechanisms underlying its pathogenicity is essential for the development of new therapeutic strategies. For that purpose, we characterized the innate immune response, in particular the production of pro and anti-inflammatory cytokines, chemokines and cell recruitment, of two-days old Balb/c mice subcutaneously infected with a lethal dose of *E. coli* K1, a serotype associated with neonatal infections. We observed a rapid colonization of the liver and lungs of infected animals, as early as 1 hour after infection, and a late colonization of spleen and brain 12 and 18 hours post-infection, respectively. In parallel with colonization, we also observed an early IL-6 production that increases over time in liver and lungs and a late IL-1β production. The blocking of both cytokine signaling did not alter the neonatal susceptibility to infection. The levels of TNF-α and anti-inflammatory cytokines IL-10 and IL-27 did not change over time in these organs. Moreover, we observed a high influx of neutrophils in lungs and liver shortly after infection which, despite high levels of the chemokines MIP-2, KC and MCP-1, decreased to the threshold level 6 hours post-infection.

In conclusion, our results indicate that *E. coli* rapidly colonizes the host by subverting the innate immune response. Such study could reveal new potential targets and/or biomarkers of sepsis to prevent the disease caused by this bacterium.

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On the path to autologous IVD regeneration?

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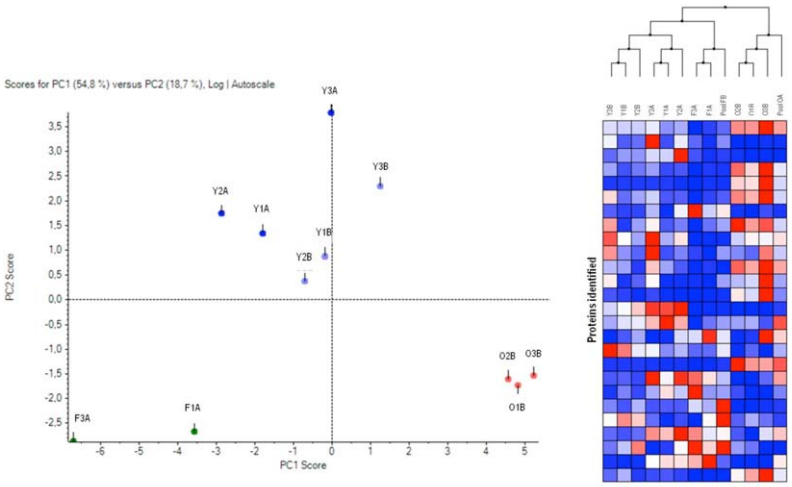
Introduction: Intervertebral disc (IVD) degeneration is often the cause of low back pain. With age, extracellular matrix (ECM) depletion occurs, leading to nucleus pulpous (NP) extrusion and IVD destruction. Concomitantly, there is a decline in viable cell populations, which in turn remain poorly characterized. Since current clinical treatments have not provided adequate solutions, we propose an in depth study in terms of NP cell sub-population characterization and ECM changes with ageing, in view of regeneration.

Materials and Methods: The first step was to characterize NP cell populations harvested from bovine IVDs, in order to better understand IVD's potential to endogenously regenerate. We started by optimizing cell isolation procedure, still not consensual amongst the literature. For that, distinct digestive enzymes (collagenase-type-I, collagenase-type-II and collagenase-type-XI) were used at three different concentrations (0.5, 1.0 and 2.0 mg/ml, respectively), for 4 and 19h. To understand the cellular effect of the digestion we evaluated the cell yield (trypan blue exclusion); cell viability/apoptosis (AnnexinV/Propidium Iodide staining, Flow Cytometry) and cell morphology (actin (phalloidin-AlexaFluor®488) and nuclei (DRAQ-5) staining, Imaging Flow Cytometry). The presence of specific cell subpopulations within NP and its phenotype was then investigated, by assessing the expression of CD29, CD44, CD45, CD146, CD34, Gly-A and Brachyury. Due to microenvironments' growing importance for regeneration, in parallel, we compared the NP ECM proteomic profile of bovine IVDs from foetus, young and old animals by quantitative iTRAQ LC-MS/MS. The candidates differentially expressed in the three age groups are being validated by Western Blot.

Results: Cell yield and viability improved, in a concentration dependent manner, with increasing collagenase activity against collagen I and II – collagenase XI rendered the highest cell yield and greatest viability. In addition, cell yield was higher for shorter digestion periods (4h). Furthermore, NP cells did not reveal major morphological changes dependent on the enzymes used. Interestingly, three viable sub-populations, with different sizes and auto-fluorescence, were consistently identified by flow cytometry, immediately after isolation. Expression of cell markers differed among these sub-populations, proving the existence of an heterogeneous cell population within the NP. Concerning iTRAQ analysis, a few interesting candidates emerged that are expressed in different proportions in fetus, young and old animals. These quantitative differences are being validated.

Conclusions: This study shows that collagenase type XI is the most efficient enzyme to be used for isolation and that different cell populations can be identified within the bovine NP. It also demonstrates that distinct ECM proteins are expressed in the IVD at different ratios depending on bovine age groups.



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A *Plasmodium* phospholipase is involved on merozoites egress from hepatocytes

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Malaria remains the most deadly parasitic disease of humans. The symptoms of the disease are caused by the multiplication of the causative agent, the *Plasmodium* parasite, inside erythrocytes. The parasite is initially inoculated into the skin of the host during a mosquito bite as sporozoites, an elongated and highly motile form of the parasite. The few tens of inoculated sporozoites reach the liver via the blood circulation, where they invade hepatocytes and each transform into thousands of merozoites, the erythrocytes infective forms. To complete its life cycle, the obligatory *Plasmodium* parasite, which multiplies only inside host cells, needs to rupture host cell membranes on repeated occasions and stages such sporozoites and merozoites. Sporozoites ability to disrupt host cell plasma membranes is required for parasites transmigration in the skin, resisting clearance from phagocytic cells and crossing liver sinusoids, while merozoites membrane rupture events are needed to egress either from hepatocytes or erythrocytes.

Plasmodium sporozoites express a phospholipase (PL), which was on a previous work proposed to be involved on sporozoites cell traversal activity. In this work we have generated a fluorescent *P. berghei* with the PL-encoding gene disrupted and new assays to directly assess sporozoites cell traversal activity. Our data indicate PbPL is not required for sporozoites cell traversal activity and surprisingly, is involved on the egress of hepatic merozoites.

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Hepcidin function in fish: two sides of the same coin

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Background: Due to the nature of their surrounding environment, fishes have developed several defense mechanisms against waterborne pathogens, with one of the first lines of defense being a number of relatively small peptides with antimicrobial properties. Among these antimicrobial peptides is hepcidin, a small cysteine- rich molecule discovered in the turn of the century, which also came to be considered the long sought key regulator of iron metabolism. Unlike most mammals, which have only one hepcidin gene, with a dual function as an antimicrobial peptide and an iron metabolism regulator, fishes can present many isoforms, which are generally attributed to genome duplications and positive Darwinian selection, influenced by host-pathogen interactions. It has been suggested that different hepcidin isoforms may assume different functions in fish, and its antimicrobial activity may be very important in fish. **Results:** To study the role of the different hepcidin genes in teleost fishes, we have isolated and characterized several hepcidin genes for a teleost fish, the European sea bass *Dicentrarchus labrax*, and evaluated variations in their expression when fish were subjected to different experimental conditions, including iron overload, anemia, bacterial infection and hypoxia. Although several isoforms were found, they could be clustered in two groups: hamp1-like, with a single isoform similar to mammalian hepcidins, and hamp2-like, with several isoforms. Under experimental *in vivo* conditions, *hamp1* was found to be up- regulated in response to iron overload and infection and down-regulated during anemia and hypoxic conditions. *Hamp2*, on the other hand, did not respond to either iron overload or anemia, but it was highly up-regulated during infection and hypoxia. In *in vitro* experiments with spleen derived leukocytes, *hamp1* was again up- regulated during iron overload and early stages of infection, whereas *hamp2* was only highly up-regulated during infection. Furthermore, one Hamp1 and three different Hamp2 peptides were commercially synthesized and tested against several pathogens. Hamp1 peptide showed no effect against any of the tested pathogens, whereas the Hamp2 peptides presented variable activity against several fish and human pathogenic Gram-negative and Gram-positive bacteria. **Conclusions:** In teleost fishes that present two hepcidin types, there seems to have been a subfunctionalization of hepcidin's functions, with hamp1 seemingly more involved in the regulation of iron levels, whereas the various hamp2 are mostly performing an antimicrobial function, with the peptides presenting different affinities. With this study, we provide an integrative insight on the functions of the different hepcidin genes in a teleost fish and demonstrate their antimicrobial potential not only for fishes, but also for other species.

High *in vitro* activity of synthetic 5-aminoimidazole-4-carboxamidrazones against *Candida* biofilms formation on nanohydroxyapatite

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Infection is currently regarded as the most severe problem associated to the use of biomaterials. A wide range of biomaterials used in clinical practice have shown to support colonization and biofilm formation by *Candida* species, with important clinical repercussions [1]. The cells within biofilms exhibit significant tolerance to antifungal therapy and have the ability to withstand host immune defenses. In addition, biofilm act as reservoirs for persistent sources of infections [2]. In a previous work, Ribeiro *et al* prepared and tested a series of 5-aminoimidazole-4-carboxamidrazones and three of them displayed strong antifungal activity on yeast [3]. Therefore, in the present study, the activity of these three novel imidazole derivatives was evaluated against *C. albicans* and *C. krusei* biofilm formation on nanohydroxypatite (nanoHA), a well-known biocompatible and bioactive ceramic [4]. Additionally, the cytotoxicity against human osteoblastic cells (MG63) was also assessed. Two approaches were applied: (1) to investigate anti-biofilm effect the components were simultaneously incubated with yeast suspension and the number of adherent cells on nanoHA surface was assessed after 24h incubation and (2) to access the mature biofilm eradication ability, 24h-biofilms of *Candida* species established on nanoHA were exposed to 5-aminoimidazole-4-carboxamidrazones for 24h and the number of remaining viable microorganisms was determined. Using both approaches, the metabolic activity of MG63 was assessed after 24h and 48h incubation with 5-aminoimidazole-4-carboxamidrazones. Concerning the results obtained, the presence of imidazole derivatives had a remarkable inhibitory effect on subsequent biofilm development by *C. albicans* and *C. krusei* on nanoHA surface. Moreover, the three tested 5-aminoimidazole-4-carboxamidrazones displayed potent *in vitro* activity against sessile yeast cells within biofilms, in a concentration-dependent way. Roughly, *C. albicans* was more sensitive to these components. The metabolic activity of MG63 cells had shown a time and concentration-dependent cytotoxic effect. Together these preliminary findings indicate that these imidazole derivatives display potent activity against *C. albicans* and *C. krusei* biofilms *in vitro*. Future studies should be carried out to estimate the potential of these components in the treatment or, even better, for the prevention of *Candida* biofilm biomaterial-associated infections.

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CD5-mediated inhibition of T cell activation: a detailed analysis of CD5 extracellular and intracellular interactions

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CD5 is a T cell surface receptor that has a modulatory role in T cell signaling. Structurally, CD5 is a transmembrane glycoprotein containing an extracellular part organized in three scavenger receptor cysteine-rich (SRCR)-type domains and an unstructured cytoplasmic tail. During antigen presentation and T cell activation, CD5 is translocated to the immunological synapse established between the T cell and the antigen-presenting cell (APC) and is able to refrain T cell signaling. Several receptors expressed in different cell types have been suggested to be CD5 ligands, but none of them has been confirmed as such. In order to uncover a CD5 ligand and characterize the functional consequence of the CD5 - CD5 ligand interaction, we have assembled recombinant CD5 with streptavidin-phycoerythrin, resulting in a tetrameric form of CD5 ($CD5_{tet}$) suitable for detecting very low affinity interactions with putative ligands. We have screened over 20 human cell lines of different origins for $CD5_{tet}$ binding, and only the monocytic cell line THP-1 and erythroleukemia K562 cells were positive. Although no definitive identification of the CD5 ligand was made possible, we have confirmed that the CD5 ligand is of protein nature since proteinase K treatment abrogated the CD5tet interaction with the ligand-positive cells. Furthermore, the mannosidase I inhibitor kifunensine did not induce any reduction of $CD5_{tet}$ binding to either THP-1 and K562 cells, excluding the involvement of the ligand's complex sugars in mediating the interaction. In the intracellular part and upon T cell receptor triggering, CD5 is phosphorylated with very rapid kinetics and couples to signaling inhibitors. One such effector is the SH2 domain-containing phosphotyrosine phosphatase, SHP-1. However, the precise mechanism or the residues involved in SHP-1 coupling to CD5 have not been fully clarified. We have now identified specific tyrosine-containing sequences within the cytoplasmic tail of CD5 that are necessary for signaling inhibition during T cell-APC recognition, and removal of these amino acids results in the loss of inhibition mediated by CD5. In particular, a peptide containing pY429 binds with very high affinity to recombinant SHP-1. Removal of this tyrosine residue from CD5 expressed at the surface of JTAg cells results is the cancellation of inhibition and in the generation of high levels of intracellular calcium, whereas in JTAg cells expressing wild-type CD5, calcium signaling remains low. We are currently addressing the interaction of phosphorylated tyrosine residues of CD5 with the SH2 domains of scaffold proteins and adaptors to identify other important CD5 partners that regulate signaling and the translocation of CD5 to the immunological synapse during antigen presentation.

Searching for peritonitis causing agents in the microbiota of Peritoneal Dialysis patients: analysis of possible routes of infection

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Objectives: Peritoneal dialysis (PD) is a home-based renal replacement therapy, currently treating 11% of the global dialysis population across 130 countries worldwide. Despite prevention strategies, peritonitis remains a leading complication of PD, accounting for more than 10% of deaths among this population. In our center there is a low but persistent number of peritonitis every year (1 peritonitis/29 months) being *Streptococcus* (24%) and *Staphylococcus* (11%) responsible for most cases of Gram+ peritonitis and *Pseudomonas* (16%) the most common organism in Gram - peritonitis. Fungal peritonitis (3%) is rare but its treatment is usually very difficult. The aim of this work was to characterize possible sources of peritonitis agents. The oral and nasal cavity and the skin surrounding the catheter exit-site of PD patients were studied and correlated with PD-related infections.

Methods: A group of 44 PD patients of S. João Hospital was studied. Microbiological analysis of saliva, nose and catheter exit-site comprised the isolation and identification of *Staphylococcus*, *Pseudomonas* and *Candida*. The following strategies were employed for microbial identification: 1) *Staphylococcus* spp.- growth in Mannitol Salt medium, morphological features by microscopic visualization after gram staining and catalase test Multi-test system API 32 STAPH; 2) *Pseudomonas* sp.- Cetrimide selective agar 10 mL/L glycerol, morphological features by microscopic visualization after Gram staining and oxidase test. 3) *Candida* spp.- growth in CHROMagar CandidaTM® medium and sequencing analysis of Internal Transcribed Spacer.

Results: PD patients had an average age of 45±14 years. The mean duration of PD was 11.6±15.6 months, and 29.5% had a previous peritonitis episode. *Staphylococcus* species most commonly associated with these infections were *S. aureus*, *S. warneri* and *S. epidermidis*. *Staphylococcus* were detected in 95% of salivary samples, 82% of nasal samples and 48% of catheter exit-site samples. *Pseudomonas* colonization was detected in only one of the evaluated samples (from saliva). PD patients presented fungal colonization in saliva (8%) and catheter exit-site (2%) whereas no nasal colonization was detected.

Conclusion: *Pseudomonas* and *Candida* species presented low prevalence whereas *Staphylococcus* species presented high prevalence among the evaluated PD patients. Also, the most common Staphylococcus species found in the saliva, nasal cavity or catheter samples of PD patients corresponded to the species mostly associated with peritonitis episodes, however, no direct relationship between normal microbiota and peritonitis occurrence were found. Further studies are clearly necessary.

Key words: Peritoneal Dialysis, Peritonitis, Microbiota.

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The global distributions of alleles associated with dengue infection and design of a predictive genetic risk model

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Climatic change and globalisation are introducing the virus and vector of dengue infection, from tropical and subtropical regions into the northern hemisphere. Nowadays, Latin America and Southeast Asia are the main endemic regions of the globe, while curiously Africa remains largely free of dengue outbreaks, although the virus and the vector are present across the continent, as testified by the reported infections of foreigners visiting or working there. Recent genetic evidence collected in admixed populations is supporting the fact that African-ancestry confers resistance to dengue infection, but possibly also allele frequencies of genes conferring susceptibility to dengue can be significantly different in Africa. In fact, allele frequencies vary across the globe following in general a model of isolation by distance (geographically closer populations have similar allele frequencies) with centre in Africa, the place of origin of our species – hence, African populations have the most different frequencies.

Some alleles have been shown to be associated with dengue infection in Latin American and Southeast Asian populations, and some of these associations have been tested through functional assays. Based in that information, we selected 13 SNPs across eight genes and designed a chip in order to genotype them in several populations from across the globe. We sampled around 1,500 individuals, and collected information from the 1000 Genomes database and available populations in literature, summing up around 4,700 individuals genotyped for each of the 13 SNPs.

To visualize the geographical distribution of each dengue associated allele, we are constructing interpolation maps of their frequencies by using the "Spatial Analyst Extension" of ArcGIS software. We are also designing a genetic risk model for these 13 SNPs based on odds ratios estimated in case-control studies performed in diverse endemic populations. The genetic risk formula will then be applied to the genotypes observed in the worldwide populations, allowing to infer the genetic risk of dengue infection conferred by these 13 SNPs across the globe. These results are of importance for the public health authorities in the north hemisphere and may help explain the low observed risk of African populations.

Unraveling the involvement of *Leishmania infantum* NDH2 and mFRD enzymes in mitochondrial NADH oxidation

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Trypanosomatids are flagellated parasitic protozoa responsible for several infectious diseases that affect humans and animals, namely leishmaniasis, Chagas's disease and sleeping sickness. The mitochondrion of trypanosomatids arises as a highly interesting research topic as it presents a number of unique traits and thus, potential drug targets. Unlike most eukaryotes, these organisms contain a single, elongated and highly branched mitochondrion with a very unusual genome (kDNA) and divergent metabolic pathways. Moreover, aside from the canonical complex I, trypanosomatid parasites have additional ways of oxidizing mitochondrial NADH as through the activity of fumarate reductase (mFDR), alternative NADH dehydrogenase (NDH2) and glycerol-3-phosphate dehydrogenase, although the relative contribution of these different systems for the maintenance of mitochondrial redox balance and in ROS production is yet unclear.

To investigate the role of NDH2 and mFRD proteins, within the mitochondrial respiratory chain, we studied their cellular localization and characterized the respective overexpressing strains (OE-NDH2 and OE-mFRD). Both enzymes are predicted as mitochondrial proteins by *in silico* analysis. A combination of indirect immunofluorescence studies and western blot analysis of protease accessibility upon digitonin fractionation and carbonate extraction confirmed that both proteins are located in the mitochondrion associated with the inner membrane. Moreover, NDH2 and mFRD proteins are expressed in both promastigote and amastigote stages of *Leishmania*.

The biochemical characterization of NDH2 and mFRD enzymes is being a challenging task since their expected activities as NADH oxidizing devices are being difficult to identify in *L. infantum*, even upon their overexpression. To circumvent this unexpected setback, metabolic alterations resulting from overexpressing NDH2 and mFRD were evaluated by oxygen consumption studies in comparison to the wild type. The results demonstrate that overexpression of NDH2 increases basal oxygen consumption with higher activities of complex II and of enzymes that feed electrons into the first entry point of the respiratory chain (including NDH2). The higher complex II activity is usually associated to an increase in the Krebs cycle activity and a decrease in glycolytic flux of the parasites. Furthermore, the respiratory activity of OE-mFRD parasites displays decreased sensitivity to KCN, a complex IV inhibitor, probably coupled to a higher oxygen consumption that diverged into ROS production by mFRD. OE-mFRD basal respiration is similar to that observed in the wild type strain being sensitive to complex II inhibitors and almost not inhibited by rotenone, an inhibitor of the proton-pumping complex I.

Helicobacter pylori activates the EphA2 receptor in gastric epithelial cells to modulate the angiogenic response

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Background: *Helicobacter pylori* is a Gram-negative bacteria that persistently colonizes the human stomach, establishing a chronic infection in more than half of the world's population. All infected individuals display chronic gastritis, and a subset of them will develop more severe gastric diseases, such as peptic ulcer disease, and gastric cancer. *H. pylori* targets various host cell surface receptors, including receptor tyrosine kinases (RTKs) such as c-Met, EGFR, and ErbB2/Her2, altering their downstream signaling pathways and modifying key cellular functions. Alterations in these and others signaling pathways have been implicated in the pathogenesis of *H. pylori*. The Eph (erythropoietin-producing human hepatocellular carcinoma) receptor family, although constituting the largest sub-family of RTKs, remains to be studied in the context of bacterial infections. Eph receptors are critical regulators of important cellular processes, including cell-substrate adhesion, cell migration, and angiogenesis. EphA2, one member of the Eph receptor family, is primarily found in adult human epithelial cells and is highly expressed in many cancers, in which its expression is associated with the progression of these carcinomas. **Aims:** To investigate whether *H. pylori* can target members of the Eph receptor family. **Results:** We found that *H. pylori* activates the EphA2 receptor early upon infection of MKN28 gastric epithelial cell line, which in turn leads to its down-regulation at later time-points of infection. No alterations of the EphA2 mRNA levels were found upon infection as assessed by quantitative real-time PCR. Using the EphA2 receptor down-regulation, observed at later time-points of infection, as a readout of the early receptor activation, we found that *H. pylori* targets the EphA2 receptor in several other gastric cells lines, such as AGS, AGSEcad, and NCI-N87 cell lines, independently of the endogenous expression of E-cadherin. Furthermore, we found that EphA2 receptor down-regulation is not strain-specific, and is independent of the major *H. pylori* virulence factors, using for infection of MKN28 cells, respectively, different *H. pylori* clinical isolates, and the *H. pylori* 60190 *wild-type* strain and its isogenic *ΔcagA*, *ΔcagE*, and *ΔvacA* mutants. Moreover, small interference RNA (siRNA)-mediated silencing of EphA2 in MKN28 cells reduced its angiogenic capacity in the *in vivo* chorioallantoic membrane (CAM) angiogenic assay. **Conclusions:** Our observations constitute the first description of *H. pylori* targeting one receptor of the Eph receptor tyrosine kinase family, the EphA2 receptor, with effect in the angiogenic response of gastric cells to *H. pylori* infection. **Relevance:** The discovery of novel molecules that are activated by *H. pylori* infection might disclose new targets for therapeutic intervention in gastric cancer.

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A novel biocomposite for bone regeneration

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This work aimed at designing a synthetic construct that mimics natural bone extracellular matrix through innovative approaches based on simultaneous type I collagen electrospinning and nanophased HA (nanoHA) electrospraying using non-denaturating conditions and non-toxic reagents. Morphological results assessed by scanning electron microscopy and atomic force microscopy (AFM) showed a mesh of collagen nanofibers embedded with crystals of HA, which fiber diameters within the nanometer range (30 nm), thus significantly lower than those reported in the literature, over 200 nm. The mechanical properties assessed by nanoindentation using AFM exhibited elastic moduli between 0.3 and 2 GPa. Fourier transformed infrared spectra analysis confirmed the collagenous integrity as well as the presence of nanoHA in composite. The network architecture allows cell access to both collagen nanofibers and HA crystals as in natural bone environment. The inclusion of nanoHA agglomerates by electrospraying to type I collagen nanofibers improved adhesion and metabolic activity of MC3T3-E1 osteoblasts. This new nanostructured collagen-nanoHA composite holds a great potential for bone defects healing or as a functionally membrane for guided bone tissue regeneration and in treating further bone diseases.

Characterization of *Leishmania* extracellular vesicles: insights into their immunoregulatory role in the establishment of the infection

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The use of secretion pathways for effector molecule delivery by microorganisms is a trademark in pathogenesis. Recent emphasis has been given to the release of extracellular vesicles (EVs) by the parasite *Leishmania*, the etiological agent of leishmaniasis. The absence of vaccines and satisfactory treatments associated to this disease demand a better understanding of the infectious process. The evasion of the immune response is critical for infection establishment. Our hypothesis is that EVs could be implicated in these early evasion mechanisms by acting on immune cells. In this sense, the immunological potential of EVs and vesicle depleted exoproteome (VDE) has been tested, *in vitro* and *in vivo*, on mouse models.

EVs and VDE were prepared from stationary promastigotes as recently described by us and further characterized. Subsequently, an air pouch model was used to resemble the biological environment of the first stages of infection, allowing the study of local cell recruitment. Mouse's backs were inflated and subsequently injected with EVs or VDE. At 6h after inoculation, animals were euthanized, the pouch exudate collected, and the presence of different cell populations studied by flow cytometry. For *in vitro* studies bone marrow-derived dendritic cells (DCs) and macrophages were used. The expression of several activation markers and cytokines was studied, by flow cytometry/ELISA, after 24h of incubation with EVs or VDE. Then, the state of responsiveness of the cells was also evaluated by re-estimulation with TLR-ligands.The promastigote secreted EVs are associated with protein markers traditionally found in exosomes, containing also genetic material. Nonetheless, evidences of a heterogeneous vesicle population hints at distinct origins. At the evaluated time point, EVs consistently recruited higher numbers of DCs and eosinophils when compared with VDE or an inflammatory stimulus as LPS. *In vitro*, both EVs and VDE are able to diminish the response capacity of DCs and macrophages to exogenous stimulus.

Leishmania released EVs might promote a specific immune cell recruitment and subsequent anergy, contributing to the survival of the parasite in the early stages of the infection.

The exoproteome of two morphologically and metabolically different cyanobacteria

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The bacterial cell envelope has deserved an enormous attention from the scientific community throughout the years. As the bacterial contact surface with the surrounding environment and the first protection barrier this multilayered structure has been one of the favorite targets for the search, development and investigation of substances that can impair bacterial cell division, essentially of those that are pathogenic. However, more than a simple protection barrier, the bacterial cell envelope plays crucial roles in biologically important processes such as facilitating bacterial communication, recognition of and adhesion to the host, exchange of biomolecules and establishment of biofilms. The knowledge regarding the cyanobacterial cell envelope is not scarce and several reports are available addressing various aspects of its structure and composition, including inner and outer membranes protein composition, peptidoglycan structure, the periplasm protein content and its continuity between neighboring cells, lipopolysaccharide composition, and extracellular matrix. However, little is known about the protein composition outside the outer membrane. Once translocated across the outer membrane a protein can remain anchored to the membrane, associate (non)-covalently with outer membrane components, assemble into macromolecular structures on the cell surface, or be released in the surrounding environment. The subset of proteins present in the extracellular milieu, the exoproteome has not been thoroughly investigated in cyanobacteria. In the last few years, there has been be a growing interest in profiling the exoproteome of bacteria, due to the distinguished roles played by exoproteins on homeostasis, development, proteolysis, adhesion and extracellular matrix organization. In this work we have analyzed the exoproteome of two morphologically and metabolically different cyanobacteria: the unicellular non-nitrogen-fixing *Synechocystis* sp. PCC 6803 and the filamentous, heterocyst-forming and nitrogen-fixing *Anabaena* sp. PCC 7120. Making use of ESI-ion trap mass spectrometry, 55 different proteins, belonging to 18 different functional categories were identified in the *Synechocystis* sp. PCC 6803 exoproteome, while 137 proteins from 35 different functional categories were identified from the *Anabaena* sp. PCC 7120 exoproteome. Components of the S-layer and the pili structures (specifically in the case of *Synechocystis*), proteins involved in the acquisition of metals and nutrients, and factors associated to the defense mechanisms towards oxidative stress, among others, could be identified. However, a large fraction (close to 40%) of the identified proteins is “hypothetical”, “unknown” or presents no similarity to other proteins, highlighting how much is unknown in terms of cyanobacterial extracellular proteome and its possible impact on the cyanobacterial cell structure and physiology. Some of these proteins are currently being characterized and their functions unveiled. One of such proteins is HesF in *Anabaena* sp. PCC 7120 [1], which was found to play a role in filaments adhesion and aggregation in diazotrophic conditions.

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Sex steroid ablation stimulates thymic rebound through an Interleukin-7-independent pathway

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The function of the thymus progressively degenerates due to age-related alterations that affect both T-cell precursors and thymic stromal cells, particularly thymic epithelial cells (TECs). Sex steroid ablation (SSA) transiently augments the production of T-cells in the involuted thymus. Still, the mechanism underlying the thymic rebound after hormonal manipulation remains incompletely characterized.

Here, we investigate whether interleukin 7 (IL-7)/IL-7 receptor axis is required for the thymic renewal induced by androgen withdrawal. We show that, similarly to the stimulatory effect observed in the aged thymus, SSA enhances T-cell production in non-atrophic thymus through an expansion of all major thymic subsets, including early thymic progenitors (ETP). As result of increased lympho-epithelial crosstalk, the cellularity of medullary TECs (mTEC) is specifically augmented, including the Aire-expressing subset. Androgen depletion does not control the expression of IL-7 by TECs, inferring that the SSA-mediated increase in thymic activity entails IL-7-independent signals. Androgen blockage of IL-7$^{-/-}$ mice improves T-cell production in the same order of magnitude as observed in immunocompetent mice. Although the specific hematopoietic defects that result from the lack of IL-7 signalling are not overridden, we report a marked improvement of the sparse mTEC niche of IL-7 deficient mice. The enhanced thymic activity is reflected in the increased numbers of splenic naive CD4^{+} and CD8^{+} T cells found in SSA-IL7$^{-/-}$ mice. Surprisingly, SSA-mediated thymic expansion is abrogated in Il2rg$^{-/-}$ mice. Analysis of the bone marrow (BM) reveals that while the numbers of the early hematopoietic progenitors (LSK and CLP) are augmented in WT and IL-7$^{-/-}$ mice, those lineages failed to expand in Il2rg$^{-/-}$ mice.

Our results indicate that androgen withdrawal stimulates thymic activity through a mechanism that involves γc-dependent, yet IL-7-independent, signals engaged in early hematopoietic progenitors. Thus, the mobilization of BM precursors by SSA might act as chief catalyst of thymic rebound.

GWAS study in Cuban admixed population – evaluating the effect of ancestry in the genetic resistance to dengue infection

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Dengue is an emerging arboviral disease caused by infection with any of the four dengue viruses (DENV) transmitted between humans by *Aedes* mosquitoes. Dengue became a major public health problem throughout the tropical and subtropical regions, and is attracting awareness in Europe and in the United States, as climatic changes and globalisation lead to the virus and vector dispersion. Some immunological genes have been reported as conferring susceptibility or resistance to the infection, but so far, the only global unbiased genome-wide association study was performed in the Vietnamese population. Most complex diseases are caused by rare mutations, which can differ between populations. So, susceptibility/resistance genetic pattern in Latino American populations, another main endemic region for dengue infection besides Asia, can be different. And given that Latino populations were made of European ancestry mixed with African background, they can be more informative to evaluate the genetic risk of European populations to dengue. The EU awareness in dengue infection is supported by an observation made by Cuban medical doctors of a significant increased risk of light-skinned Cubans to develop dengue infection and presenting the worse phenotype than dark-skinned Cubans.

We performed a GWAS study in the Cuban island, in patient (dengue fever and dengue hemorrhagic fever), control and asymptomatic samples collected in two regions, Havana and Guantanamo, during the 2006 outbreaks. We genotyped 274 individuals for the Illumina chip containing 2.5 million SNP. A global genome analyses allowed to confirm the African-ancestry influence in resisting dengue infection in Havana (especially in the worse phenotype: 9% of African ancestry in hemorrhagic compared with 33% in asymptomatic, and 24% in fever), but not in Guantanamo (38% in asymptomatic, 36% in hemorrhagic and 35% in fever). The population structure in both groups (especially Havana) bias simple association tests, leading to false associations. To circumvent this effect, we are applying local ancestry inference techniques (along each autosome) and selection algorithms (decrease of diversity) which allow to identify significantly different regions of the genome, which may contain genes potentially associated with the disease. As individual contribution of genes is expectably low but these tend to be associated in pathways, we are also using gene enrichment tools to detect them.

This work shows that even within an Island, the heterogeneity in admixture between population groups can introduce variability in the genetic susceptibility/resistance to dengue infection. This heterogeneity must be taken into consideration when predicting the genetic risk of a population to an infectious disease, impacting diagnosis and prognosis of the disease.

Fibrinogen implants for bone regeneration: short- and long-term *in vivo* responses

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Inflammatory response is a natural event following biomaterials implantation. The concept of "fighting inflammation" has been gradually shifting to "modulating inflammation"[1]. Our team has previously shown that the incorporation of a pro-inflammatory and pro-healing molecule, fibrinogen (Fg), in chitosan scaffolds is able to modulate the systemic immune response while improve bone regeneration[2]. In this study we addressed the potential of Fg 3D scaffolds to promote bone regeneration. Also, we investigated the local and systemic response to these scaffolds at short- and long-term time-points after implantation.

Fg scaffolds were prepared by freeze-drying method as previously described by our group for other materials2. Animal work was approved by DGAV. Each Wistar rat (3 months) suffered a femoral critical size bone defect, which remained empty or received Fg scaffolds (n=12/group). Before implantation the scaffolds were neutralized and disinfected by an ethanol series gradient and kept in sterile PBS. After 6 days (short-term) and 8 weeks (long-term) post-implantation, the animals were sacrificed. The blood (BL), lymph nodes (LN), spleen (SP) and femurs were collected. Histology, gene expression analysis of inflammatory and bone-related markers was analysed at the defect site in the remaining femurs. Immune cell populations from BL, LN and SP were analysed in FACSCanto. Pro-inflammatory cytokines in plasma were also analysed by ELISA. Statistical analysis was performed using Prism software.

At 6 days post-implantation, local gene expression analysis at the bone defect revealed a significant increase in IL-6 and IL-8 levels for both, empty- and Fg-groups of animals when compared with non operated animals, while IL-1B remained constant. An increase of VEGF and TGF-B1 was also detected. At the same time-point post-implantation, TGF-B1 concentration in plasma was significantly increased in the "empty"-group of animals, while at 8 weeks post-implantation no differences were observed between the groups of animals. The pro-inflammatory cytokines TNF-a, IL-6 and IL-17a were not detected in plasma, at either time point.

Also, Fg scaffold implantation correlated with changes on the proportions of immune cells at the systemic level. A reduction of myeloid cells ($CD11b^+$) and B cells, with a concomitant increase on T cells, was particularly significant in SP at 6 days post-implantation. Fg also led to less NK and NKT cells in BL. Interestingly, BL, LN and SP Mac-1^+ ($CD18^+/CD11b^+$) cells were significantly decreased in animals implanted with Fg scaffolds, while more TCR^{dim} T cells were found in LN of the animals with Fg implants. Moreover, BL myeloid cells ($CD11b^+$) showed an activated phenotype (MHC^{high}) in Fg implanted animals.

Histological evaluation showed that Fg scaffolds eased cell migration. The increased infiltration of connective tissue at periphery of the defect in Fg-group correlated with a decreased defect size at day 6. By 8 weeks post-implantation Fg scaffolds were replaced by bone tissue.

Fg scaffolds promote bone formation after 8 weeks of implantation. A mild inflammatory response was observed 6 days after injury, either with or without Fg implants, when compared with non-operated animals. Moreover, alterations in immune cell proportions and their activation status, at an early time-point correlate with increased bone formation at a later stage.

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Unraveling the role of Scavenger Receptors in *Listeria* infection

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Microorganisms have evolved sophisticated counter-measures, against which the host needs to respond by adjusting or improving its defenses. During infection, host cells react to the presence of microorganisms through pattern recognition receptors (PRRs) that recognize, in a broad non-specific manner, conserved pathogen-associated molecular patterns (PAMPs), leading to the activation of the innate immune system. Scavenger receptors (SRs) comprise an emergent multifunctional family of PRRs able to sense and eliminate unwanted entities. Several SRs have been implicated in bacterial recognition and appear to play key functions in antimicrobial host defense.

We aim to identify and characterize SRs involved in the host interaction with *Listeria monocytogenes* (*Lm*), a human intracellular foodborne pathogen capable to cause listeriosis. This opportunistic bacterium displays a unique competitive advantage of crossing the intestinal, blood-brain and materno-fetal barriers.

In order to discriminate a role for SRs on the cellular infectious process, we evaluated how *Lm* adhesion and invasion steps were affected by general chemical inhibition of SRs. Upon saturation of SRs, the ability of *Lm* to invade both phagocytic and non-phagocytic cells was significantly impaired, without compromising adhesion to host cells. Furthermore, chemical inhibition of SRs did not affect the invasiveness of other bacteria, suggesting an *Lm* specificity concerning the potential role of SRs on cell invasion. To narrow for candidates putatively involved on *Lm*-host interaction, we analysed SR expression profiles of several epithelial cells and macrophages, and identified SRs differentially expressed upon *Lm* infection. This analysis revealed that hMARCO and mainly hFEEL1 were highly expressed in HeLa cells in response to Lm infection. The involvement of specific SRs in the host interaction with Lm was further explored by RNAi depletion. We observed that the silencing of both hSR-A and hFEEL-1 in HeLa cells induced a decrease of Lm internalization.

Altogether, these data suggested a role for SRs in the initial steps of the *Listeria* cellular infectious process and highlight the relevance of *Lm*-SR interactions on the host response and virulence. We are now characterizing the molecular details of these interactions and their exact role on *Lm* virulence.

Effects of heavy metals on *Cyanothece* sp. CCY 0110: growth, EPS production, ultrastructure and protein profiles

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Many cyanobacterial strains can synthesize and secrete extracellular polymeric substances (EPS) that can remain associated to the cell or be released into the environment (RPS-released polysaccharides). The particular features of cyanobacterial EPS, namely the presence of 2 different uronic acids, sulphate groups and high number of different monosaccharides (up to 12), makes them promising for biotechnological applications such as the removal of heavy metals from polluted waters [1]. For the successful implementation of systems based on cyanobacterial EPS, it is necessary to unveil the pathways utilized for synthesis and export, identify the physiological/environmental factors that influence the synthesis and/or the characteristics of the polymers and characterize the interactions between the cells/EPS with the metal ions.

We evaluated the effects of the presence/concentration of several heavy metals commonly found in polluted waters (copper, lead, cadmium, lithium) in the growth/survival, ultrastructure and EPS production by the unicellular cyanobacterium *Cyanothece* sp. CCY 0110. This marine N_2-fixing cyanobacterium has its genome fully sequenced and is among the most efficient RPS producers [2]. In addition, the proteomes of cells grown in the absence/presence of an essential (Cu^{2+}) or a non-essential (Cd^{2+}) metal were compared using iTRAQ isobaric tagging technology.

We concluded that different heavy metals affect *Cyanothece* cells differently and the response triggered to cope with these metals is also distinctive, with the cells being more sensitive to Cu^{2+} (cell death at 0.2 mg/l), followed by Pb^{2+}, Cd^{2+} and Li^{+} (cell death at 70 mg/l). This is most probably related to the cells specific micronutrients requirements (i.e. essential vs non-essential metal) and the mechanisms of metal uptake/accumulation inside the cells. In general, carbohydrate production followed the pattern of growth, with RPS constituting in average 58±6% of the amount of total carbohydrates. In addition, the visible ultrastructural changes observed in cells grown in medium supplemented with metal are mainly related with thylakoid disorganization and increased intrathylakoidal spaces. The proteomes of cells exposed to Cu^{2+} revealed that cells tune down their metabolic rate, including O_2 evolution, CO_2 fixation and N_2 assimilation to invest the spare energy in the activation of metal detoxification mechanisms, which ultimately results in a remarkable recovery. In contrast, the toxic effects of Cd^{2+} accumulate over time, contributing to lower efficiency of the detoxification mechanisms compared to Cu^{2+}, ultimately resulting in cell death. The results presented can contribute to understand how the cyanobacterial cells cope with the presence and different concentrations of metals and will help to implement metal removal systems based on cyanobacteria or their isolated EPS.

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Keratins 8 and 18 are required for *Listeria monocytogenes* invasion of mammalian cells

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To promote infection pathogens interfere with crucial host intracellular pathways. In particular, host cytoskeleton components are preferential targets of infecting bacteria. The study of the cell biology of infection provided insights in the way bacteria manipulate the host cytoskeleton and revealed unsuspected functions of cellular proteins, leading to a deeper understanding of eukaryotic basic cellular processes. *Listeria monocytogenes* is a facultative intracellular gram-positive pathogen adapted to thrive in diverse environments. In humans, *Listeria* is capable to cause listeriosis, a pernicious foodborne disease, largely usurping cytoskeleton functions during host cell infection. Keratins are major components of the cytoskeleton of epithelial cells with structural and regulatory functions. Although keratins were reported to be targeted by pathogens, the molecular and functional details behind keratin involvement in bacterial pathogenesis remain largely elusive.

In this work we studied the importance of Keratin 8 (K8) and its preferential binding partner Keratin 18 (K18) in *Listeria monocytogenes* pathogenesis. Our results demonstrate that both K8 and K18, together with actin and cMet, a major *Listeria* receptor, are found enriched in the vicinity of *Listeria* invading HeLa cells. Depletion of K8 and K18 by an RNAi approach revealed that both keratins are required for *Listeria* entry in Hela cells. Likewise, InlB/c-Met-dependent internalization of *Listeria* is found to be impaired in cells depleted for K8 and/or K18. Strikingly, we found that K8/K18 depletion results in the down-regulation of cMet levels and impaired cMet signaling. Additionally, using beads coated with the InlB invasin, a listerial cMet agonist, we found that actin recruitment precedes K18 enrichment during InlB-mediated internalization in HeLa cells, suggesting that keratins may be relevant in later stages of *Listeria* uptake.

Together our results point out keratins as important players in *Listeria* pathogenesis. The molecular details of their involvement in *Listeria* infection and cMet signaling are under investigation.

Unraveling the mechanism of the Zn-mediated regulation of gene expression in *Leishmania infantum*

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Our group has previously identified and characterized the first zinc (Zn) transporter in *Leishmania infantum*, *Li*ZIP3, a protein encoded by two identical genes arranged in tandem. *Li*ZIP3 expression was shown to decrease when Zn is in excess due to the destabilization of its mRNA. This process requires the involvement of a short-lived protein that interacts with Zn-responsive element(s) within the *Li*ZIP3 3' untranslated region (UTR). Given that Zn is crucial for biological processes but toxic if in excess, we are investigating the mechanisms through which *Leishmania* maintain Zn homeostasis. We started by identifying the *Li*ZIP3 Zn-sensing element(s). For this, we produced vectors expressing luciferase under the control of the complete *Li*ZIP3 3'UTR or of the 3'UTR with deleted fragments and introduced them into *L. infantum* by electroporation. By comparing the activity and expression of luciferase in transfected parasites grown in normal and in Zn-supplemented media we mapped the *Li*ZIP3 Zn-responsive element(s) to a 116-nt long region. Further deletions on this sequence and point-mutations will be made in order to better delimitate this element(s)

Additionally, we are currently employing RNAseq to identify the set of *Leishmania* genes co-regulated by Zn as we hypothesize that co-regulated genes will share Zn-responsive elements. Consensus motives in the UTRs of these genes may be retrieved by bioinformatic analysis and, in the case of those regulated similarly to *Li*ZIP3, by comparison with the *Li*ZIP3 Zn-responsive element(s). These results may lead us not only to the characterization of Zn-sensing elements but also to the identification of regulatory proteins that bind to them.

OxyR interplays with a novel sigma factor in the regulation of the oxidative stress response and iron metabolism in *S. tsukubaensis*.

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Iron is an essential nutrient for all living organisms; however iron overload can be harmful due to the formation of hydroxyl radicals via the Fenton reaction. Given this toxicity, bacteria have developed a strict control and regulation of iron homeostasis that interplays with the oxidative stress response in oxic environments. OxyR is a hydrogen peroxide sensing LysR protein that regulates bacterial response to oxidative stress and is described to be involved in metal ion homeostasis as a response and adaptation to peroxide stress. In *Streptomyces coelicolor*, OxyR has been described as a positive transcriptional regulator of the ahpCD operon. In this work we characterize the role of the *oxyR-ahpCD* system in *S. tsukubaensis* NRRL 18488.

In silico analysis showed that *S. tsukubaensis* presents an unique genomic organization of the *oxyR* region: OxyR encoding gene is located 3.9 kb upstream from the *ahpCD* operon and three novel genes are present, including *STSU_11560* that encodes a novel extracellular sigma factor that shares 67% homology with *S. clavuligerus* extracytoplasmic function (ECF) sigma factor SigG and is divergently transcribed from *oxyR*.

Transcription analysis of the *S. tsukubaensis* wt, *Δsig*, *ΔoxyR* and *ΔahpC* strains showed a cross-regulation between OxyR and the sigma factor STSU_11560. As expected, *oxyR* and *ahpC* transcription was induced in the wild-type upon an H_2O_2 insult. Furthermore, EMSA analysis showed that OxyR binds to the *STSU_11560-oxyR* intergenic and to the *ahpC* promoter regions in both its reduced and oxidized form. However, *sig* transcription was not induced by H_2O_2 addition suggesting that the sigma factor STSU_11560 is not involved in the immediate response to induced oxidative stress by H_2O_2. Further characterization of the *Δsig* strain, defective on the sigma factor STSU_11560, revealed a hampered iron uptake although siderophore production levels in iron limiting conditions were similar to the wild-type.

We were able to establish that STSU_11560, encoding an ECF sigma factor, controls the uptake of ferrous iron in *S. tsukubaensis*. Additionally, the ferrous iron uptake and oxidative stress response are being independently regulated by Sig and OxyR, respectively.

Adaptive evolution and divergence of SERPINB3: a young duplicate in Great Apes

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The superfamily of serine protease inhibitors (SERPIN) plays a crucial role in the regulation of several physiological processes, including inflammation and immune response. *SERPINB* genes undergone an expansion throughout vertebrate evolution by a series of duplication events, which led to a functional diversification of the inhibitory repertoire of these proteins. This is the case of the two recent duplicates *SERPINB3* and *B4*, located in the human 18q21.3 gene cluster, which are known to control the activity of different cysteine and serine proteases. Both SERPINB3/B4 seem to play a major role in cell protection against excessive proteolysis and in the immune response to pathogens. Also, SERPINB3 is overexpressed in some squamous epithelial cancers and is up-regulated in several autoimmune diseases. However, despite their key role in many physiological processes the mechanisms of action of SERPINB3/B4 remain fairly unknown. Therefore, we aim to assess the coevolution of SERPINB3/B4 and their target proteases in the view of understanding the evolutionary forces that shaped the accelerated divergence of these duplicates. A phylogenetic analysis of several primate *SERPINB3/B4* genes places the duplication event in a Hominoidae ancestor (30 Mya) and the emergence of *SERPINB3* in Homininae (9 Mya). Moreover, we found evidence of strong positive selection throughout primate evolution for SERPINB4/B3 tree and their target proteases, cathepsin L2 (CTSL2) and G (CTSG) and chymase (CMA1), which suggests that SERPINB3/B4 functional divergence could be related with adaptive evolution. Additionally, in the Homininae clade we detected a perfect match in the adaptive evolution of *SERPINB3* and cathepsin S (CTSS). The projection of the sites under positive selection in the three-dimensional inhibitor-protease complex shows that most of them are located in regions crucial for the inhibitory activity. In this way, a large percentage of the sites under positive selection are located in the inhibitor reactive center loop (RCL) and in the loops near the enzyme catalytic pockets, at the inhibitor/protease interface. Such regions are recognized for their crucial role in the functional specificity of the inhibitor and in the substrate affinity of the enzyme, thus suggesting a co-evolution scenario for SERPINB3/B4 and their target proteases. Furthermore, SERPINB3/B4 have similar expression patterns and share several regulatory cis-motifs that are best explained by a neofunctionalization model of the evolutionary fate of duplicates. Overall, these findings point to a functional divergence of *SERPINB3/B4* duplicates towards a broader and/or more specialized repertoire of protease inhibitors. Also, the ability of SERPINB3/B4 to inhibit exogenous proteases, released by infectious agents, may also suggest an accelerated evolution driven by host-pathogen interactions.

Leishmania infantum in vivo infection modulates host iron metabolism proteins as a possible mechanism to gain access to iron

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Leishmania infantum, a causative agent of canine and human visceral leishmaniasis, must acquire essential nutrients to thrive in their hosts. Crucial to multiple biological functions, iron is one of such nutrients. As a means to obtain this essential metal, pathogens employ a diversity of mechanisms which often include the modulation of the host iron metabolism.

In this work we aimed at analysing whether, *in vivo*, *L. infantum* modulates the expression of iron host metabolism proteins as a possible mechanism to gain access to such a vital nutrient. For that, we collected, at different time-points, the liver and spleen of mice infected with *L. infantum* and assessed the expression of the host iron exporter ferroportin-1 (FPN-1) and the iron metabolism regulator hormone, hepcidin.

So far, we uncovered that in *L. infantum*-infected kupffer cells the localization of FPN-1 is modified. Moreover, the altered FPN-1 localization in infected macrophages appears to occur through a mechanism that is not mediated by increased levels of hepcidin. Overall, our data unravels that the presence of the parasite has an impact on the iron metabolism of the host and indicates a possible strategy of *Leishmania* to obtain iron.

Confocal Microscopy Distribution Studies of Fluorescent Iron Chelators with Antimicrobial Activity in BM derived Macrophages

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Bacterial resistance to currently used antibiotics is one of the most important problems for human society and the development of new antimicrobial therapies remains crucial. The process of iron acquisition represents a pathway which can be successfully targeted by novel therapeutic tools and iron restriction has been shown to improve the outcome of a number of infectious diseases.

We previously reported the inhibitory effect of rhodamine-derived iron chelators on intracellular growth of *Mycobacterium avium* [1]. These previous results showed that the chelation of iron is a determinant but not sufficient property for antimicrobial activity, since biological activity seems to be dependent on the fluorophore structure. Chelators bearing the rhodamine B isothiocyanate fluorophore (MRH7, MRB7) proved to be more effective than those derived from carboxy-rhodamine (MRH8, MRB8). The results obtained in biophysical studies performed in liposomes provided evidence that the more active compounds strongly interact with the lipid phase while the less-active do not, suggesting that intracellular distribution might determine antimicrobial effectiveness.

In this work we report results of the confocal microscopy distribution studies performed in bone marrow derived macrophages including experiments in which markers for the various cellular organelles have been used. We found that the distribution of the chelators within macrophages is similar for all the red chelators but the uptake is different for the compounds bearing fluorophores 7 or 8 as revealed by the different amount of ligand necessary to visualize the compounds. All fluorescent chelators studied seem to have access to phagosomes and to mitochondria but not to early or late endosomes. MRH7 does not appear to colocalized with *M. avium*- containing phagosomes and seems to access endoplasmic reticulum compartments (Fig. 1). We believe that the differences in the hydrophilic/lipophilic balance justify these results as the chelators MRH7 and MRB7 have a higher affinity for the lipid phase.

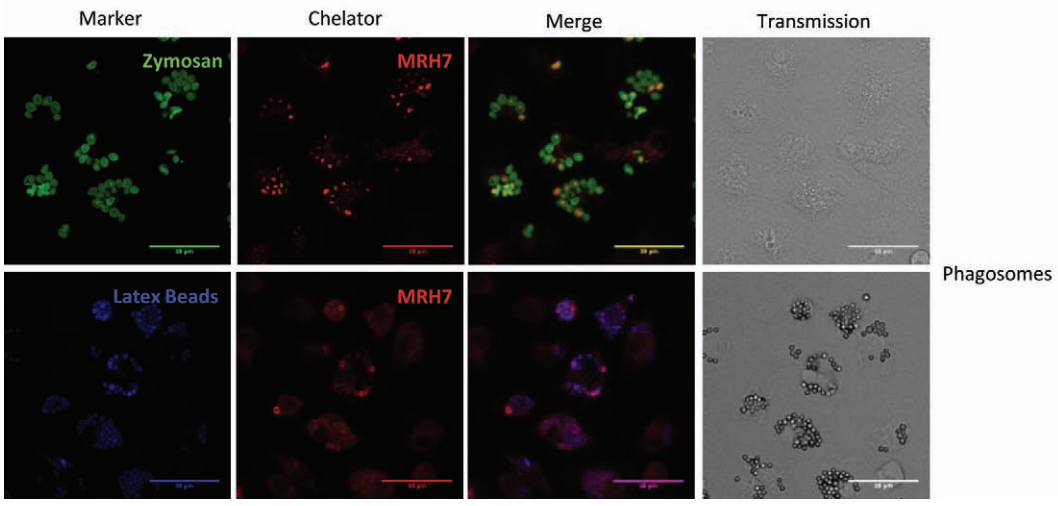

IMAGE 1: Representative confocal microscopy images of intracellular colocalization studies of MRH7 chelator in BM derived macrophages.

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ROS as a key players in the *Streptomyces natalensis* morphological differentiation.

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Introduction. *Streptomyces* are aerobic Gram-positive bacteria that undergo a complex life cycle. After spore germination a first vegetative mycelium (MI) is formed characterized by highly septated hypha and an active primary metabolism; upon nutrient starvation certain hyphal compartments undergo programmed cell death (PCD) and a second vegetative mycelium (MII) arises from the viable compartments; along with the formation of MII a shift from primary into secondary metabolism occurs and the developmental program is activated; then the superficial layer of MII differentiates into aerial mycelium, while the innermost mycelium goes through a second round of PCD; finally spores are formed after aerial hypha septation.

The identification and characterization of proteins that are part of both the stress response network and *Streptomyces* development program shows that these physiological processes are intimately connected. Additionally there are a number of studies that associate the accumulation of reactive oxygen species (ROS) to physiological stresses and to PCD. In this study we provide further evidences regarding the role of ROS in streptomycetes morphological differentiation.

Results. *S. natalensis ΔkatA1* and *ΔcatR* were constructed, defective on the monofunctional catalase and on the fur-like repressor that controls *katA1* expression, respectively. Both strains presented morphological differentiation impaired phenotypes; *ΔkatA1* displayed a bald phenotype, while *ΔcatR* formed scarce aerial mycelium.

Characterization of the mutant strains showed an early activation of molecular defences against hydrogen peroxide and free ferrous iron (Fe^{2+}) as well as an induction of primary metabolism. The early activation of these protective mechanisms in *ΔkatA1* can be related to an adaptive response to the lack of an important antioxidant mechanism, the monofunctional catalase. Concerning antioxidant defences activation in *ΔcatR*, the over-expression of the catalase *katA1* was a direct consequence of *catR* deletion. Curiously, *ΔkatA1* vegetative mycelium seems to be kept at the MI stage, since the typical highly septated hyphae are still observed at late stages of cultivation. This correlated with an extension of vegetative mycelium life-span in *ΔkatA1*. Additionally, the transcription of key development related genes was repressed in both mutant strains.

Conclusion. The results suggest that the morphology impaired phenotypes observed in the S. natalensis mutant strains, *ΔkatA1* and *ΔcatR*, are the consequence of an inhibition or retardation of PCD associated with ROS homeostasis disruption. This study highlights the importance of intracellular ROS levels in the morphological development program of *S. natalensis*.

The genetic suppression of NRF2 causes age-related hepatic fibrosis in a mouse model of hereditary hemochromatosis

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Introduction: HFE-associated hereditary hemochromatosis (HH) is the most common genetic disorder of iron overload in Caucasians. When untreated, it can lead to iron accumulation in tissues, especially in the liver, with secondary organ damage attributed to oxidative stress. Patients have increased risk of hepatic fibrosis, cirrhosis and hepatocellular carcinoma, often after the fourth decade. Most HH patients are homozygous for a common mutation in the HFE gene (C282Y) and organ disease occurs only in a minority of C282Y homozygotes due to genetic/environmental factors that remain mostly undetermined. We have recently reported that the transcription factor NRF2, a key regulator of cell/organism adaptation to oxidative stress, is important for hepatocyte viability during exposure to acute dietary iron overload (Silva-Gomes *et al.* 2014). **Objective:** To evaluate if the genetic disruption of NRF2 would prompt the development of spontaneous liver damage in $Hfe^{-/-}$ mice (an established model of HH).

Materials and methods: $Nrf2^{-/-}$ and $Hfe^{-/-}$ mice on C57BL/6 genetic background were crossed to generate double knock-out mice ($Hfe/Nrf2^{-/-}$). Wild-type, $Nrf2^{-/-}$, $Hfe^{-/-}$ and $Hfe/Nrf2^{-/-}$ female mice were sacrificed at age 6, 12 or 18 months for evaluation of blood parameters and liver pathology. **Results:** $Hfe^{-/-}$ and $Hfe/Nrf2^{-/-}$ mice displayed features similar to those described in human C282Y homozygotes: elevated serum iron, transferrin saturation, mean corpuscular volume and mean corpuscular hemoglobin; mild parenchymal iron overload; and low liver hepcidin levels. Despite having hepatic iron stores similar to $Hfe^{-/-}$, $Hfe/Nrf2^{-/-}$ mice presented significantly higher numbers of necroinflammatory foci and apoptotic (TUNEL-positive) cells, which increased with age and associated with gradual iron accumulation in hepatic macrophages. In 12- and 18-month old mice, the latter formed large aggregates ('siderotic nodules'). Myofibroblasts (ACTA2+) observed in the damaged areas associated with substantial amounts of collagen fibers involving the liver parenchyma (hepatic fibrosis) which increased with age. **Conclusion:** In the absence of NRF2, the mild hepatic iron overload observed in the mouse model of HH associates with increased necroinflammation and hepatic fibrosis along with ageing of HFE knock-out mice.

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Silva-Gomes *et al.* 2014 J Hepatol. 60(2):354-61.

The Dynamics of Fibroblasts/ECM in Neonatal Cardiac Injury

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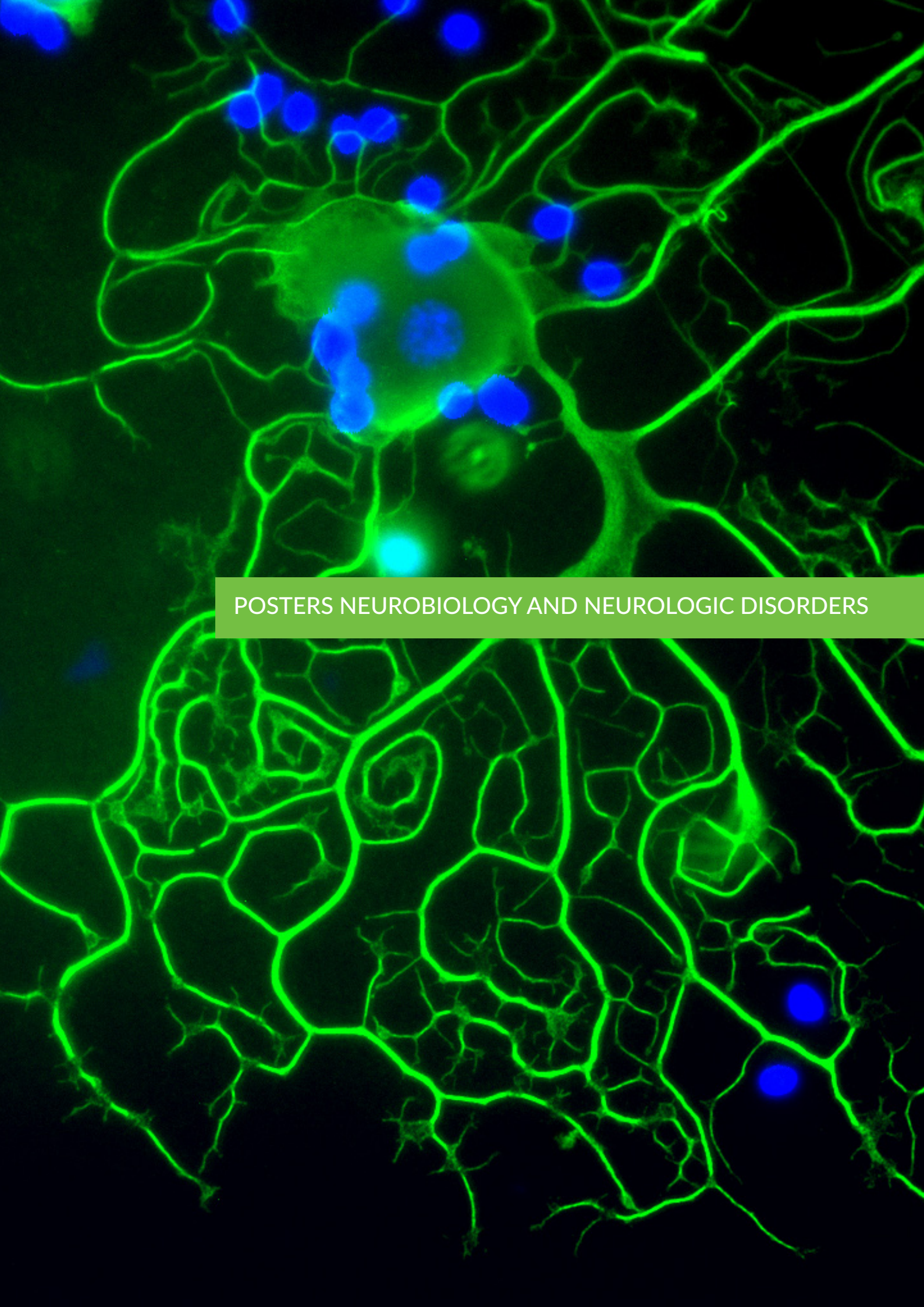
Cardiac diseases remain the major cause of morbidity and mortality worldwide. This is the ground for an increasing number of studies on the characterization of the heart response to injury and on the development of new therapies to functionally restore the damaged myocardium. Although the mammalian heart displays regenerative ability during the first 6 days following birth, after this period, if injured it triggers the formation of a non-functional fibrotic scar. However, the signals that determine the development of a reparative (adult) or regenerative response (neonate) are largely unknown. The premise of our Team is that the transient regenerative capacity of the heart is related to specific organ changes during this ontogenic period. The herein work focus on the dynamics of cardiac fibroblasts and that of the extracellular matrix during ontogeny and following injury in the neonate. We demonstrate that fibroblasts, herein defined as $CD31^{-}CD45^{-}Ter119^{-}CD90^{+}$ population colonize the heart following birth and also that the cell-surface signature of the stromal compartment also changes throughout post-natal life. We also provide evidence that the regenerative neonatal response to apex resection involves the recruitment of inflammatory cells, fibroblast activation, extracellular matrix production and neo-vascularization. High Content Analysis (HCA) revealed that the removed tissue was, at least in part, re-established by the proliferation of resident cardiomyocytes. Echocardiographic functional evaluation of the injured hearts revealed functional restoration at 21d post-lesion which correlates with tissue recovery at the histological level.

Overall this work describes the microenvironmental alterations, with particular emphasis on fibroblasts and extracellular matrix, that are triggered following neonatal apex resection and that culminate on the functional and histological restoration of the organ.



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Trimethyl Chitosan-Based Nanoparticles Intracellular Trafficking and Transfection: a Bioimaging Study

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Introduction: Trimethyl chitosan (TMC) is a promising gene delivery vector for biomedical applications. Due to the quaternization of the primary amines, independently of the pH, TMC in solution is positively charged[1]. In the context of the application of non-viral vectors for gene therapy, biomaterial degradation and the fate of the resulting by-products are important issues to take into consideration in the design of delivery systems. TMC enzymatic biodegradation occurs between acetylated monomers and is mostly affected by the degree of acetylation (DA)[2]. In this work, we explored the impact of the DA on the enzymatic degradation and biological properties of TMC-based nanoparticles. Nanoparticle internalization, co-localization with intracellular organelles and transfection efficiency were evaluated in TMC nanoparticles with different DAs.

Materials and methods: TMC with 43.3 kDa, 11% DA and 30% degree of quaternization (TMC_{11}) was used to prepare TMC/DNA complexes with different N/P ratios (N - moles of quaternized amines and P - moles of phosphate groups) in 20 mM HEPES buffer with 5% (w/v) glucose at pH 7.4. CH with similar Mn and DA was used as control. The original TMC was deacetylated and subsequently re-acetylated to achieve the different DAs[3]. To evaluate TMC nanoparticles enzymatic degradation a competition assay was performed[3]. Nanoparticles internalization and intracellular localization were evaluated by imaging flow cytometry between 0.5 h to 24 h of incubation with ND7/23 cells (a neuronal cell line). Finally, the impact of nanoparticle biodegradation rate on transfection efficiency was assessed 72 h post-transfection, using GFP as a reporter gene.

Results and discussion: TMC_{11} was successfully deacetylated (DA 2%, TMC_2) and subsequently re-acetylated (DA 17%, TMC_{17}), with no significant alteration of the polymer molecular weight. TMC-DNA complexes were efficiently prepared, with an average diameter of 200 nm, irrespectively of the N/P ratio and polymer DA. The enzymatic degradation profile of the TMC-nanoparticles is in accordance with the literature for TMC solutions: a higher DA led to a higher polymer degradation rate[2]. The internalization efficiency of TMC nanoparticles was evaluated by imaging flow cytometry. No major differences were observed between TMCs when analyzing the number of particles associated to the whole cell (percentage of membrane bound and cytoplasm localized nanoparticles). However, by discriminating these two counterparts one observe significant differences between TMCs.

Nanoparticles co-localization (pDNA stained with YOYO-1) with nuclear DNA (stained with DRAQ5) was also evaluated. At 0.5 h of incubation, already in 4% of cells, nanoparticles were co-localizing with nucleus, irrespective of the DA. From 4 h onwards, a significant difference was seen between TMC's with different DAs, where TMC_{11} mediated the delivery of a higher quantity of pDNA to the cell nucleus. TMC_{11} also presented the highest levels of cell transfection (% of cells expressing the delivered reporter gene).

Conclusions: TMC nanoparticle biodegradation is influenced by the polymers DA. The 24 h bioimaging study of the nanoparticles intracellular route presented herein discloses important data about the process of intracellular trafficking indicating that biodegradation, hence associated DNA availability, is a key feature for the overall transfection process.

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ObserVaPark: an Observational study on Vascular Parkinsonism

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Vascular parkinsonism (VP) is a secondary parkinsonism related with vascular lesions that comprises a distinct clinicopathological entity. It presumably accounts for 4,4 – 12 % of all cases of parkinsonism. The majority of studies are retrospective and don't use the available operational criteria which would increase future comparability research.

The aim of this study is to prospectively follow-up, during a period of 2 years, patients with vascular parkinsonism whose diagnosis has been made in the past 4 years and in those newly diagnosed. The objective is to describe clinical, radiological and therapeutic features of Vascular Parkinsonism and compare them to a group of patients with Parkinson Disease. Secondary objectives comprise characterization of non-motor symptoms in vascular parkinsonism, evolution (number of falls, cognition), sleep disorders, vascular parkinsonism diagnosis differences between the available two rating scales, and prevalence of side effects.

The study will be conducted in a single center (Serviço de Neurologia do Centro Hospitalar de Trás-os-Montes e Alto Douro) from 2015 until 2017. Four neurologists, 2 of them specialized in Movement Disorders, will evaluate patients. The research team will also include a neurorradiologist and a neuropsychologist. Clinical evaluation will be undertaken at the usual month intervals. All patients will be evaluated with brain imaging and scored using the following scales: Modified Rankin Scale, The Barthel Index (Portuguese version), Mini-mental State Examination (Portuguese version), The Montreal Cognitive Assessment (MOCA, Portuguese version), Parkinson´s disease questionnaire (PDQ-39, Portuguese version), Unified Parkinson's Disease Rating Scale (UPDRS) – I, III, IV and V (ON); II and VI (ON and OFF), Non-motor symptom assessment scale for Parkinson's Disease (NMSS), Parkinson´s Disease Sleep Scale (PDSS-2), Berg Balance Scale and Falls efficacy scale (Portuguese version).

This is the first study addressing this condition in Portugal, where cerebrovascular disease has high incidence rates.

We are looking for research groups with a similar research profile interested in participating in a project that includes patients with movement disorders.

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Cell-matrix constructs for central nervous system regeneration using embryonic stem-derived neural stem/progenitor cells embedded in fibrin hydrogels

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Nervous system disorders are one of the main causes of disability worldwide. Transplantation of neural stem/progenitor cells (NSPCs) represents a strategy to treat these pathologies either by replacing lost cells or by releasing neurotrophic factors. Biodegradable hydrogels are being explored as vehicles for NSPC transplantation, by allowing a more favorable microenvironment for cell survival and neuronal regeneration. This work aims to develop a fibrin (Fb)-based gel for the delivery of embryonic stem (ES) cell-derived NSPCs into the injured central nervous system. Previously, we established the optimal cell culture conditions to culture ES-NSCs in Fb. Here, ES-NSPCs were embedded in Fb and characterized in terms of cell yield, phenotype, secretion of extracellular matrix (ECM) proteins and matrix metalloproteinase activity (MMPs). The effect of fibrinogen concentration on neuronal differentiation and cellular outgrowth in Fb was also assessed.

Fb gels were prepared by mixing equal volumes of a thrombin solution containing CaCl2 and aprotinin, and a plasminogen-free human fibrinogen solution containing factor XIII and dissociated ES-NSPCs. The cell-matrix constructs were cultured for periods up to 14 days, following a protocol for neuronal differentiation. At day 14, the constructs were processed for immunolabeling of phenotypic markers and ECM proteins (fibronectin and laminin) or for the quantitative analysis of cell phenotype by flow cytometry. MMP activity in cell-conditioned media was evaluated at day 4, 8 and 14 of cell culture, using gelatin-zymography, while matriptase (MTP) expression was determined after 14 days of culture in protein extracts by Western blotting and analyzed by densitometry.

ES-NSPCs in Fb proliferated more during the first week of culture (5-fold increase) and less at the second (2-fold increase). Through imaging analysis, we found that cell-matrix constructs were mainly composed by a heterogeneous population constituted by NSPCs and neurons, expressing nestin and β-III tubulin, respectively. Additionally, at day 14, the presence of mature neurons expressing GABAergic and dopaminergic phenotypic markers was also observed. A network of fibronectin and laminin could be also detected, at this time point. Zymograms revealed that NSPCs embedded in Fb secrete MMP-2 and MMP-9, the latter being detected at a later period of cell culture. MTP expression increased significantly upon cell embedment in Fb, though no significant differences were found among the different points of cell culture investigated. Fb gels prepared with the highest fibrinogen concentration tested were found to inhibit cell outward migration from NSPC spheroids and to hamper differentiation along the neuronal lineage.

Dissociated ES-NSPCs in Fb proliferate and establish neuronal networks. Furthermore, Fb-cultured NSPCs are able to secrete ECM components and MMPs which will possibly contribute to the degradation of Fb in an *in vivo* scenario. Moreover, Fb gels with different viscoelastic properties were shown to affect ES-NSPC behavior.

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Dissecting the role of profilin-1 in axon formation, growth and regeneration

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Emerging evidence suggests that regulators of cytoskeleton dynamics might be attractive targets to control axon formation, growth and regeneration. Specifically, actin dynamics in the growth cone is thought to play a major role in generating the force required for axon extension. Although actin is well recognized as a key player in axonal growth, how different actin-binding proteins control its dynamics is still not fully understood. Using the conditioning lesion, a model in which the axonal regeneration capacity of spinal dorsal column axons is increased following a priming lesion to the sciatic nerve, we determined that the actin-binding protein profilin-1 (Pfn1), is increased in regenerating axons. Besides, acute *in vitro* ablation of Pfn1 precluded axon formation in hippocampal neurons and significantly decreased neurite outgrowth in DRG neurons. Collectively, these evidence point to a critical role of Pfn1 during early neuritogenesis and axonal regrowth following injury. To unravel the importance of Pfn1 during axonal regeneration, we generated mice with an inducible neuronal deletion of Pfn1 using cre-lox technology. Thy1cre$^{+/-}$Pfn1$^{fl/fl}$ neurons, lacking Pfn1, displayed impaired actin dynamics and defective neurite outgrowth upon a conditioning lesion. Besides, *in vivo* regeneration of both peripheral and central axons tended to be diminished. Thus, Pfn1 emerges as a potential determinant for axonal regeneration capacity. To dissect the molecular mechanisms that underlie Pfn1 function in neurons, we used overexpression of Pfn1 mutants in *in vitro* neurite outgrowth assays. In this system, a phospho-resistant mutant of Pfn1, the constitutively active form of the protein, strongly enhanced neurite outgrowth. Hence, specific modulation of regulatory regions of Pfn1 could represent a useful approach for future therapeutic applications. Moreover, this work also revealed that Pfn1 is capable of linking actin and the microtubule cytoskeleton by interfering with intracellular signaling namely with the PI3K/AKT/GSK pathway.

In summary, our work uncovered Pfn1 has an important determinant in axon formation growth and regeneration.

Demyelination precedes but is unrelated to the differential axonal degeneration in central nervous system of plasmalogen-deficient mice

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Nervous tissue is highly enriched in plasmalogens, a class of ether-phospholipids, whose biosynthesis initiates in peroxisomes and is required to attain the high levels of these phospholipids in myelin and neurons. The importance of plasmalogens to human health is emphasized by the severe clinical presentation of Rhizomelic Chondrodysplasia Punctata (RCDP). The neurological involvement in RCDP, with impaired myelination, seizures and intellectual disability combined with the observation of plasmalogen deficiencies in several neurodegenerative disorders underscores the role and function of these phospholipids in neurons and myelinating glia. In Gnpat knockout (KO) mice, we show that lack of plasmalogens causes myelination defects in the central nervous system. In spinal cord, a defect in plasmalogens leads to an adult onset demyelination without axonal loss or loss of oligodendrocytes. Astrocytosis and microgliosis were also a feature in the white matter of spinal cords from KO mice. In optic nerve, the lack of plasmalogens initially impairs myelination (dysmyelination) and is followed by a rapid and progressive demyelination. Surprisingly, dysmyelination or demyelination of optic nerves in KO mice was not accompanied by axonal pathology. Unlike spinal cord, optic nerves from plasmalogen-deficient mice did not exhibit microgliosis or axonal damage, only revealing astrocytosis during the late stage demyelination. In summary, contrarily to the characteristic demyelination and axonal loss that occurs in the peripheral nervous system of KO mice, the CNS axons are preserved despite a severe demyelination. Besides revealing the importance of plasmalogens for myelination and myelin maintenance, our results highlight that in the presence of oligodendrocytes, loss of myelin has minimal consequences to axon integrity despite differential susceptibility of neurons to demyelination.

A pilot study about alternatives for adult zebrafish anesthesia

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Background: The importance of zebrafish on research has been increasing. The most used anesthetic, MS222, may induce stress1 and the search for a better alternative is a current need to improve the use of zebrafish model.

Objectives: To study the quality of anesthesia and recovery induced by different concentrations of propofol alone and in combination with different concentrations of lidocaine.

Materials and methods: Forty-four 1.5 years old zebrafish were used to test 6 different anaesthetics protocols placed in the water-bath (27.8±0.69ºC): 0.0025mg/ml (1), 0.005mg/ml (2), and 0.0075mg/ml (3) of propofol and 0.0025mg/ml of propofol combined with 0.05mg/ml (4), 0.1mg/ml (5) or 0.15mg/ml of lidocaine (6); n= 5. After the loss of the tail pinch reflex, fishes recovered.

Results: Time to lose equilibrium and to recover were similar between groups. Group with the lowest propofol concentration took more time to stop reacting to light touch than group 3 (p= 0.026) and to lose tail pinch reflex than group 3, 5 and 6 (p≤ 0.0019). High concentration groups (3 and 6) had more fishes that didn't react to observer approach, 1h post-anesthesia. 24h post-anesthesia, the activity was lower in the group 1 than in control. There were 37.5% of mortality in groups 1 and 5, 16.7% in groups 2, 3 and 6, and no fish died in group 4.

Conclusions: Concerning quick anaesthesia, recovery and mortality, the combination 2.5µg/ml of propofol with 50µg/ml of lidocaine seems to provide quality anesthesia in zebrafish.

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Experiments on temporal learning: from intra and interdimensional gradients to a generalization-based account of complex behavior

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The present work investigated whether phenomena observed in more familiar stimulus dimensions also occur in the temporal domain. Three studies were conducted. Study 1 and Study 2 investigated the shape of intradimensional temporal generalization gradients and addressed whether the context effect, a complex result in the temporal domain, which seems to suggest relational learning, could be explained by the combination of such intradimensional temporal generalization gradients. In these studies pigeons learned the discriminations 1s-Red/4s-Green and 4s-Blue/16s-Yellow. Afterwards they had to choose between B and G. The results showed that the preference for G over B increased with sample duration – the context effect, and that this preference could be predicted from the individual (asymmetric) gradients for G and B. Study 3 explored the effects of interdimensional training on the temporal generalization gradient. Pigeons learned to discriminate the presence and the absence of a t-s sample. Subsequently, when the sample duration was varied, the pigeons showed negative-exponential-like temporal generalization gradients from the beginning of testing, suggesting that temporal control can be established without explicit discrimination training along the temporal continuum. Together the three studies showed that phenomena observed in more familiar stimulus dimensions also occur in the temporal domain. The present results also have implications for current models of timing.

Role of RNA-binding proteins in oligodendrocyte differentiation

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Localization and translational control of mRNAs play a key role on the development and differentiation of different cell types. Such control is mediated by RNA-binding proteins (RBP), which are the main regulators of RNA metabolism. In the central nervous system (CNS), myelin is formed by oligodendrocytes (OL), which extend cytoplasmic processes to myelinate axons. We hypothesize that similarly to axons local translation regulation of mRNA is also important for OL development, axonal targeting and survival. To gain deeper insight into the mechanisms regulating the outgrowth of OL processes, we have performed a comparative analysis of the RNAs present in the soma and processes of OL precursor cells (OPC) by RNA-Seq. Our data revealed an enrichment of mRNAs related to cytoskeletal dynamics and translation in OPC processes. Of particular interest, we found that the mRNA levels of RBPs involved in translation such as poly(A)-binding protein (PABP) cytoplasmic 1 (PABPC1) and Y box binding protein (YBX1), were enriched. Furthermore, we created a bioinformatics tool (PBSFinder), to investigate putative binding sites for RBPs, present within the 3`untranslated region of mRNAs important for OL processes extension such as PABPC1 and YBX1, which we found to be differentially expressed at mRNA and protein level during OL differentiation. Interestingly, shRNA mediated depletion of PABPC1, inhibited OL differentiation and altered YBX1 mRNA expression. Taken together our results suggest that in OPC/OL the translation of several mRNAs is locally controlled and that PABPC1 and YBX1 play a role in this regulatory mechanism.

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Cytoskeleton regulation in methamphetamine-induced changes in neuronal morphology

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Methamphetamine (METH) is a highly addictive psychoactive drug. METH abuse results in neurotoxicity mediated by oxidative stress, increased glutamate release, persistent depletion of dopamine and serotonin contents, inflammation, and neuronal death. Chronic exposure to amphetamines also changes neuronal morphology, increasing the length of dendrites, density of dendritic spines and number of branched spines, in a long-lasting and region specific way. Although the molecular mechanisms underlying such changes are not well characterize, it is expected that they involve the regulation of cytoskeletal related proteins, and the local mRNA targeting and translation of cytoskeletal proteins in the neuronal processes. RhoGTPases are highly expressed in the nervous system, and key regulators of neuronal morphology. The negative regulation of RhoA, and its effector Rho-associated protein kinase (ROCK), or positive regulation of rac1, promotes neurite formation and outgrowth, dendritic spine formation and maintenance, and synaptogenesis. We aim to explore the molecular mechanisms by which chronic exposure of neurons to methamphetamine induces changes in neuronal morphology, looking particularly at neurite outgrowth, dendritic arborization, and density of dendritic spines.

In this work we show that incubation of cultured hippocampal neurons with a single dose of 10 or 100 µM METH for 24 hours causes an increase in total neurite length in a dose dependent way, without promoting mitochondrial toxicity neither neuronal death. We also analyzed the activation pattern of RhoA and Rac1 in hippocampal neurons after METH incubation by western blot and the topographical activation by FRET technology.

Based on these effects of METH in neuronal morphology, we will further investigate METH action using low density hippocampal neuronal cultures, which will allow a more detailed analysis of dendritic arborization and dendritic spines. As the morphological effects might be related to changes in cytoskeletal related proteins, as well as require neurite localization and translation of mRNA, we will compare the soma and neurite transcriptome of cultured hippocampal neurons exposed to METH with neurons not exposed. We will select the mRNA mainly located at neurites, paying particular attention at mRNAs translating for proteins putatively involved in RhoA/ROCK signaling and other RhoGTPases suh as rac1, in cytoskeletal remodeling and in the regulation of the translation machinery. We will then validate targets identified in the RNA seq, and characterize the function of selected validated proteins in neurite extension, dendritic arborization and spine formation.

We expect to identify molecules promoting neurite outgrowth, dendritic arborization and spine formation under METH exposure, ultimately to develop strategies to revert some of the chronic effects of METH, and to promote neuronal regeneration.

Sodium vitamin C co-transporter-2 (SVCT-2) regulates the pro-inflammatory signature of microglial cell

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Ascorbate is an antioxidant with important functions in the central nervous system (CNS). It is transported in a sodium-dependent manner by a plasma membrane transporter (sodium vitamin C co-transporter-2; SVCT-2). Microglial cells are a population of specialized resident immune cells of the CNS. When over-activated (with a characteristic M1 polarization) these cells trigger neuroinflammatory responses and potentially neural cell death. Here, retinal inflammation models, we investigated the importance of SVCT-2 in microglia physiology and the effect of pro-inflammatory stimulation (exposure to lipopolysaccharide, LPS) on the cellular localization of SVCT-2.

Primary microglial cell cultures and the N9 microglial cell line were used. *In vivo* retinal inflammation was induced by intravitreal injection of LPS or ischemia-reperfusion injury. The expression and localization of SVCT-2 in microglia were evaluated by immunocytochemistry confocal microscopy, biotinylation assay and western blotting. Lentiviral vectors were used either to knock down SVCT-2 expression by shRNA delivery, or to overexpress SVCT-2 in microglia.

Confocal microscopy analysis indicated that SVCT-2 is expressed in microglia in both cell cultures and the retina. LPS treatment decreased the membrane content of SVCT-2 triggering its degradation. Intravitreal injection of LPS triggered an inflammatory response in the retina and it also decreased the surface content of SVCT-2 in microglia. Knocking down SVCT-2 in microglial cells displayed was sufficient to induce M1 polarization, with increased reactive oxygen species and nitric oxide production, nuclear translocation of NF-kappa-B, TNF release and phagocytosis, Overexpression of wild-type SVCT-2 in microglia abolished the LPS-triggered M1 polarization.

Overall, these data suggest an essential role for SVCT-2 in controlling microglia M1 polarization and neuroinflammation.

Targeted Gene Delivery to Peripheral Sensorial Neurons mediated by Trimethyl Chitosan Nanoparticles

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In this work we propose the development of a targeted, non-toxic and efficient carrier for gene delivery to neurons. The novelty relies on the quaternization of chitosan (CH) to improve its transfection ability under physiological conditions. To characterize the nanoparticle (NP) internalization efficiency, we propose the use of ImageStream®, a technique that allows the distinction between membrane-associated and internalized NPs, which has been a challenge in the field. For the design of targeted NPs, we are exploring the use of molecular recognition force spectroscopy (MRFS) as a tool to define the optimal ligand density in the functionalized NPs able to mediate maximal neuron cell-specific interaction[1].

Trimethyl chitosan (TMC) was used to prepare TMC:DNA nanoparticles (NPs) in 20 mM HEPES buffer with 5% (w/v) glucose, pH 7.4. CH based NPs prepared in 5 mM acetate buffer (pH 5.5) were used as control. NPs were characterized in terms of size, zeta potential, complexation (SybrGold assay) and transfection (FACS) efficiency. The internalization and intracellular localization at different time-points (0.5 to 24h) of labeled NPs was assessed by ImageStream® (Amnis, Millipore). To obtain targeted NPs, the non-toxic fragment of tetanus toxin (HC) was grafted to the binary complexes. For MRFS experiments either HC or NPs with different densities of HC were tethered to an atomic force microscopy (AFM) tip via flexible PEG linkers. Measurements were carried out using a PicoPlus (Agilent) AFM instrument in both neuronal and non-neuronal cell lines.

TMC NPs present smaller sizes and lower polydispersity compared to CH ones. Also, they present a 2-fold higher complexation efficiency of pDNA, confirming its higher stability at physiological conditions. TMC NPs were found to be more abundant in cell cytoplasm at all time-points tested, while CH complexes, especially at earlier time-points, were predominantly associated to the cell membrane. The amount of vesicle-loaded NPs per cell was also higher for TMC complexes. Moreover, DNA from TMC NPs was found to have a higher co-localization with cell nuclei. Accordingly, the transfection efficiency of TMC NPs was 15-fold higher than the one mediated by CH.

The uptake of the targeted NPs significantly decreased in non-neuronal cell lines, indicating an increased specificity to neuronal cells. To maximize the targeting potential of the NPs, MRFS studies are being conducted. The obtained results confirm the interaction between HC and its receptors on neuronal cells. The MRFS measurements conducted with HC-blocked neuronal cells or in non-neuronal lines resulted in a significant drop of the interaction, confirming the specificity of the binding. Similar experiments are now ongoing, using tips bearing NPs with different densities of HC.

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How age-at-onset in FAP ATTRV30M is affected by the triad of TTR, gender and genetic modifiers?

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Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominant systemic amyloidosis. A wide variability in age-at-onset (AO) has been uncovered, including among Portuguese patients [17-82 yrs]. Early (≤40) and late-onset (≥50) cases are not separate entities, often coexisting in the same family, with offspring showing anticipation - a much earlier AO than their affected parent.

Our aims were: 1) to study anticipation in a larger number of kindreds than assessed before and to gain more insight into parent-of-origin effects; 2) to search for genetic modifiers, within or closely linked to the TTR locus, that may in part explain the observed AO variability; 3) to study candidate-genes, unlinked to TTR, that may also be influencing AO.

From the UCP-registry, we analyzed 926 parent-offspring pairs with well-established AO to study anticipation. So far, we ascertained 616 DNA samples of around 140 FAP families affected with the ATTRV30M mutation. We also analyzed 63 control trios.

Haplotype analysis is underway, using intragenic SNPs for extended haplotypes. Eight tagging SNPs were selected, with a minor allele frequency (MAF) of 0.1% and covering ~26 Kb around the TTR locus.

We selected two candidate-genes (*APCS* and *RBP4*), which were assessed in a previous study in Portuguese patients, although variation between generations was not taken into account. Four tagging SNPs and also the 5 SNPs previously described were studied. Genotyping is being performed by SNaPshot, sequencing and RFLP.

We found that women had a statistically significant higher AO than men, either for daughters vs. sons or mothers vs. fathers. Furthermore, mother-son pairs showed a larger anticipation while the father-daughter pairs showed only residual anticipation.

When we focused on genetic modifiers, we found two haplotypes in the *TTR* locus, which are more frequent in mutation carriers than non-carriers. We also found a haplotype that is only present in early-onset patient's group when compared with the late-onset patient's group.

Regarding candidate genes, we confirmed that some of the SNPs in *APCS* and *RBP4* genes are significantly associated with AO variation.

These findings confirm anticipation as true biological phenomenon. Furthermore, both parents and offspring's gender were found to be highly significant factors for anticipation. Importantly, in a larger sample of Portuguese families, we expect to disentangle the results found for these genetic modifiers in FAP ATTRV30M AO variability, within and between families which may have an important impact in genetic counselling.

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Assessment of amylin‘s nociceptive effect in an animal model of neuropathic pain

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Background and aims: Amylin is a novel neuropeptide that is expressed in C-peptidergic neurons of dorsal root ganglia. Various studies suggest a role for amylin in nociception, and its expression is down-regulated following sciatic nerve transection. Here we aimed at clarifying the effect of amylin‘s administration in neuropathic pain.

Methods: Neuropathic pain was induced by the Spared Nerve Injury (SNI) model and its development was assessed by the von Frey, pin prick, acetone (at days 7, 10, 14, 17 and 21) and cold plate (at day 21) acute pain behavioral tests. The effect of acute (day 14) and chronic (via minipump, day 14-21) subcutaneous amylin administration was evaluated both on nociception and on c-Fos expression in the ipsilateral dorsal horn of L4-5 spinal cord segments.

Results: SNI animals showed the expected signs of allodynia and hyperalgesia comparing to sham-controls, as evaluated by the behavioral tests. Acute amylin administration produced no significant effects. Chronic subcutaneous administration of amylin significantly and progressively increased the paw withdrawal duration upon the Acetone test of cold alodynia comparing to SNI control (saline-treated) animals. The number of c-Fos immunoreactive neurons in spinal cord dorsal horn (laminae I-VI) was slightly altered by the amylin treatment.

Conclusions: Data revealed that chronic subcutaneous administration of amylin worsened cold allodynia in SNI animals, suggesting that amylin may have a pro-nociceptive role in neuropathic pain. Small changes in c-Fos expression indicate that amylin is likely to modulate the activity of the spinal neuronal circuitry.

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Neuronal targeting and retrograde axonal transport of TMC-based nanoparticles in primary neurons

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Introduction: The delivery of therapeutic drugs targeted to sensorial neurons of the dorsal root ganglia (DRG) and spinal motor neurons offers the prospect of developing new clinical interventions against peripheral nerve and central nervous system disorders. In this context the understanding of how neuronal receptors are involved in trafficking and retrograde transport can open the way to the design of novel nanoparticle systems for drug delivery targeted to specific neural populations and able to be administrated via the peripheral route (minimally invasive). Here, we propose the use of microfluidics to assess the targeting capability and retrograde transport of DNA vectorized by nanoparticles based on trimethyl chitosan (TMC) functionalized with the non-toxic carboxylic fragment of tetanus toxin (HC), which is described to have tropism to neuronal populations [1]. Using compartmented neuronal cultures, treatments can be applied separately to cell bodies and axonal ends, and their effect or mode of action can be individually analyzed.

Materials and Methods: To track TMC-based nanoparticles by microscopy we labelled the TMC with rhodamine. To investigate the axon targeting and retrograde transport of these nanoparticles, we cultured DRG or spinal motor neurons in compartmented microfluidic chambers and incubated the nanoparticles in the axonal compartment. Neurons were identified through immunocytochemistry and imaged at 30 minutes and 24h incubation timepoints, using a confocal microscope.

Results and Discussion: After 30 minutes of incubation these nanoparticles exhibited specific binding to primary sensorial and motor neurons and at 24h incubation nanoparticles were detected on the neuronal cell body compartment. The HC-functionalization conferred to these vectors the capability of being transported retrogradely from the axonal tip to the neuronal cell body. In contrast, those nanoparticles non-functionalized with the HC fragment were not able to be trafficked to the neuronal cell body.

Conclusions: Here, the use of a microfluidic system allowed neuronal compartmentalization and provided the ability to control each cellular microenvironment in both spatial and temporal manner in a way that more closely resembles an *in vivo* model.

These results open up the clinical potential of these nanoparticles for minimally invasive administration of peripheral and central nervous system-targeted therapeutics in motor and sensorial neuron diseases.

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The feedback loop between bone and the peripheral NPY neuronal pathway in bone injury setting

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Neuropeptide Y (NPY) system was demonstrated to be an important regulator of bone mass formation. Recently, we also showed that NPY-ergic activity within sensory nervous system is targeted during the initial steps of bone repair, supporting its involvement in the nervous system response in scenarios were bone homeostasis is challenged. However, so far nothing is known regarding the molecular signals responsible for the feedback loop from the injured bone to the nervous system. In order to clarify this feedback loop, as a first approach, the response of the sensory nervous system to soluble factors released during bone injury was investigated through an *ex vivo* experiment where mice dorsal root ganglia (DRG) were treated with plasma from femur-defect mice. The obtained results showed that plasma from femur-defect and sham-operated mice induces different NPY and Y1 receptor expression in the DRG, supporting that soluble factors produced specifically in response to bone defect are capable of modulating the NPY-ergic activity. During bone repair, the simultaneous presence of an intense re-innervation process and an inflammatory reaction supports that inflammatory response may provide key factors for the feedback from injured bone to the nervous system. Therefore, following, in order to explore the putative involvement of pro-inflammatory cytokines, we start by investigating the local inflammatory reaction to bone injury. The obtained results showed that femur-defect mice presents higher expression levels of IL-10 and TNF-α as compared to sham-operated mice, suggesting specificity in the inflammatory response to bone injury and allowing the identification of candidates capable of mediating the feedback loop from injured bone to peripheral NPY neuronal pathway. Overall, the obtained results support that bone injury releases factors able to modulate the NPY-ergic activity in the sensory nervous system, and IL-10 and TNF-α were identified as putative mediators in this communication.

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Characterization of Casz1 expression throughout mouse dorsal root ganglia and spinal cord development

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Paired-related homeobox protein like 1 (Prrxl1) is a homeodomain (HD) transcription factor (TF) critical for the development/maintenance of both peripheral and spinal cord dorsal horn nociceptive neurons, dedicated to the processing of pain input (1, 2). A previous study characterized Prrxl1 transcriptional program in developing mouse DRG and dorsal spinal cord (SC) and found *Casz1* as an interesting putative Prrxl1direct transcriptional target gene specifically in dorsal SC. In the present study, we validated that Casz1 is directly regulated by Prrxl1 in dorsal SC. *Casz1* encodes an evolutionary conserved C2H2 Zn-finger transcription factor. In *Drosophila*, *Casz1* homolog, *Cas*, functions to regulate neural fate (3-7). Recently, Liu *et al.*(8-10) studies have shown that Casz1 is important for cell cycle exit as well as for some aspects of differentiation in several human neuroblastoma cell lines. We characterized here, for the first time, the expression pattern of Casz1 throughout mouse DRG and SC development. We found that Casz1 is expressed in virtually all DRG neurons. In dorsal SC, Casz1 appears to be exclusively expressed in the glutamatergic population exhibiting a transient expression pattern in the most superficial *laminae* throughout embryogenesis. However, further studies will be conducted in order to address *Casz1* role in developing dorsal spinal cord.

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Phosphoproteome analysis to elucidate the role of the ceramide-activated protein phosphatase Sit4p in mitochondrial function and lifespan in yeast

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The *Saccharomyces cerevisiae* Sit4p is the catalytic subunit of a ceramide-activated serine-threonine protein phosphatase, with multiple cellular roles as regulation of cell cycle progression, mitochondrial function, stress resistance and life span [1-3]. Sit4p is activated in cells lacking Isc1p, a protein with a role in sphingolipid metabolism. Moreover, SIT4 deletion abolishes the premature ageing and oxidative stress sensitivity of *isc1Δ* mutants by suppressing mitochondrial dysfunction [3]. As such, the identification and characterization of Sit4p downstream targets will extend our knowledge on Sit4p function and provide insights into the mechanism linking sphingolipid metabolism and modulation of mitochondrial function.

With this objective, *S. cerevisiae* BY4741 (wild type) and *sit4Δ* cells were used to search for changes in expression of proteins and in phosphorylation levels that may allow to identify targets of Sit4p-mediated dephosphorylation. Proteins from mitochondrial fractions were analyzed by two dimensional gel electrophoresis. Phosphoproteins were then detected by Western blotting and identified by mass spectrometry.

The analysis of the phosphoproteome in whole cell extracts revealed 9 proteins differentially phosphorylated in *sit4Δ* cells. Among those, Atp2p, the subunit β of the F1Fo ATP synthase complex, exhibited the most dramatic increase in phosphorylation. *ATP2* deletion decreased oxygen consumption of *sit4Δ* cells in post-diauxic phase but not in exponential phase. The hydrogen peroxide resistance of the *sit4Δ* mutant remained unaffected while the chronological lifespan was strongly decreased. To characterize the impact of Atp2p phosphorylation on the *sit4Δ* phenotypes, the phosphorylated Ser or Thr residues were identified by sequencing the phosphopeptides and will be mutated into Ala (phosphoresistant) or Glu (phosphomimetic) residues.

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Role of Transthyretin in Aβ peptide brain efflux – insights from *in vitro* and *in vivo* studies

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Transthyretin (TTR) has been associated with Alzheimer's disease (AD) and a novel concept of TTR stability has been established *in vitro*, as a key factor in TTR/Aβ interaction. Small compounds - TTR stabilizers - usually non-steroid anti-inflammatory drugs (NSAIDs) bind to the thyroxine (T_4) central binding channel, increasing TTR tetrameric stability and TTR/Aβ interaction. The effects of one of the TTR stabilizers identified as improving TTR/Aβ interaction - iododiflunisal (IDIF) - in Aβ deposition and other AD features, has been evaluated *in vivo* using an AD mouse model. The results showed that IDIF administered orally decreased brain Aβ levels and deposition and improved cognitive function associated to the AD-like neuropathology in this mouse model. Aβ levels were reduced in plasma and presented a trend for reduction in the cerebrospinal fluid (CSF), suggesting TTR promoted Aβ clearance from the brain and the periphery.

This project aims at investigating the influence of TTR in Aβ transport across the blood- brain barrier (BBB), using a cellular model – human cerebral microvascular endothelial cells (HCMEC/D3). We perform internalization studies of labelled (FAM-Aβ1-42) or non-labelled Aβ peptide (Aβ1-42) in the presence and absence of TTR, using flow cytometry, fluorescence microscopy or fluorescence analysis of cellular lysates.

We also performed permeability studies using cell monolayers established in transwells filters, and measured Aβ transport from the basolateral (brain) to the apical (blood) side, showing that only TTR added to the basolateral chamber is able to promote Aβ efflux, when comparing to TTR added to the apical side. Finally, we performed a preliminary *in vivo* study, using mice carrying only one copy of the TTR gene ($TTR^{+/-}$) and animals without TTR ($TTR^{-/-}$). We injected FAM-Aβ1-42 intracranial and our results indicated that, 30 minutes post injection, brains from $TTR^{+/-}$ mice retained less peptide than $TTR^{-/-}$ animals, further supporting a TTR role in Aβ brain efflux.

Fabaceae genomic and transcriptomic analyses support a different origin of the gametophytic self-incompatibility from that of Rosaceae and Solanaceae.

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Fabaceae species are important in agronomy and livestock nourishment. Fabaceae species have a long breeding history, and thus, as it happens with most species relevant to agriculture, most cultivars have lost self-incompatibly (SI), a genetic barrier to self-fertilization. Nevertheless, to improve legume crop breeding, crosses with wild SI relatives of the cultivated varieties are often performed. Therefore, it is fundamental to characterize the SI systems present in Fabaceae. The current hypothesis is that Fabaceae SI is *RNase* based. We characterized *T2-RNase* genes present in the *Trifolium pratense*, *Medicago truncatula*, *Cicer arietinum*, *Glycine max*, and *Lupinus angustifolius* genomes. Except for *T. pratensis*, all species are self-compatible (SC). Nevertheless SSK1 genes, described only in species presenting GSI, are here identified in all species. Thus, in these SC species, the *S*-locus genes may be present. In *T. pratense*, *M. truncatula* and *C. arietinum* we identify *S-RNase* lineage genes that in phylogenetic analyses cluster with Pryrinae *S-RNases*. In *M. truncatula* and *C. arietinum* genomes, where large scaffolds are available, these sequences are surrounded by F-box genes that in phylogenetic analyses also cluster with *SFBB* and *SLFL* -like genes. In *T. pratense* the *S-RNase* lineage gene shows no expression in the style, where the GSI reaction occurs. Moreover, the *M. truncatula* and *C. arietinum* S-*RNase* like and *SFBB* like genes, are expressed in other tissues not involved in GSI. We also obtained a style transcriptome of *Cytisus striatus*, a species that shows significant difference on the percentage of pollen growth in self and cross-pollinations. We have identified *SSK1* genes and *S-RNase* lineage genes in *C. striatus*. Nevertheless, none of these genes behaves as a *S-RNase* gene. Thus, we find no evidence for Fabaceae GSI being determined by *S-RNases* belonging to the Rosaceae and Solanaceae *S*- lineage genes.

Delivery of antisense oligonucleotides in a fibrin hydrogel for spinal cord injury

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Axonal regeneration following spinal cord injury (SCI) is jeopardized by the inhibitory environment of the lesion site and the activation of cell intrinsic pathways. The modulation of the expression of molecular cues involved in axonal growth inhibition, such as the RhoA pathway, could be achieved using gene therapy tools. In this context, single-stranded antisense oligonucleotides (ssAONs) can constitute a promising approach to promote axonal regeneration [1]. In the present work we propose to use a local and sustained delivery of the ssAONs into the SCI site using a fibrin (Fb)-based injectable system. Here, we investigated, *in vitro* and *in vivo*, the cell behavior in the presence of Fb-containing ssAONs.

Fb gels containing 6 and 14 mg/mL fibrinogen (Fib), thrombin (Thr), $CaCl_2$ and aprotinin (Ap) were prepared. The Fb structure was analyzed by confocal microscopy using fluorescently-labeled fibrinogen (FL-Fib). E18 rat dorsal root ganglia (DRG) were embedded in Fb containing non-functional free ssAONs (6 μM) or trimethyl chitosan-complexed ssAONs nanoparticles (NPs; 0.15 and 0.075 μM) for 2-7 days. Neurite outgrowth was quantified by β-III tubulin staining. A spinal cord hemisection was performed in female Wistar rats. The lesion site was filled with Fb gel containing 14 mg/mL FL-Fib and free ssAONs (1 μM). The lesion site was covered by a membrane of poly(trimethylene-ε-caprolactone) (11:89 mol%). Cell uptake of the fluorescent-labelled ssAONs was analyzed by immunohistochemistry.

Higher neurite outgrowth was observed in gels containing free ssAONs, for both Fib concentrations, while NPs did not affect outgrowth. Interestingly, gels with free ssAONs showed increased Fb density and decreased porosity, suggesting that the ssAONs are affecting both gel polymerization and fibrinolysis. Concerning the ssAONs uptake, while free ssAONs were found in the cytoplasm and nuclei of neuronal and non-neuronal cells at 2DIV, NPs were mainly found at the periphery of DRG explants at 3DIV. The lower rate of NP penetration in the DRG explants might be due to the net positive charge of the NPs (+8 mV) and/or cell adhesive properties of chitosan. At 7DIV, the NPs were already observed in the center of DRG, mostly at the cell's cytoplasm, suggesting that longer time is needed for a broadened NP distribution within the DRG. *In vivo*, the ssAONs delivered within Fb gels were mainly found at the lesion site at 5 days post-injury. They were also observed across the caudal side of spinal cord. Immunohistochemistry data showed both a nuclear and cytoplasmic distribution of the ssAONs. The cell populations that internalized the ssAONs are currently being assessed.

In conclusion, the ssAONs reached nuclei and cytoplasm of DRG cells when delivered within Fb, establishing the use of DRG explants as a model to assess the bioactivity of functional ssAONs. The *in vivo* delivery of ssAONs within an Fb-based system improved its retention within the lesion site, without compromising its internalization by cells.

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Endovanilloid modulation of RVM-mediated pain processing during painful diabetic neuropathy

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Most research on the transient receptor vanilloid type 1 (TRPV1) in pain facilitation and pain transmission has been focused at peripheral and spinal level of the somatosensory system. The involvement of endovanilloids in supraspinal pain modulation was recently demonstrated for the periaqueductal grey (PAG)-rostroventromedial medulla (RVM)-spinal pain pathway. Activation of TRPV1 at the ventrolateral PAG was shown to tonically influence RVM neuronal activity, inhibiting pain by activating the anti-nociceptive OFF cells activity. TRPV1 was also described in RVM neurons, however its role on pain processing remains totally unclear. Besides its role on tonic pain processing, supraspinal TRPV1 has been associated with adaptative changes occurring through the somatosensory system during neuropathic pain and changes in TRPV1 expression were already reported in the PAG of streptozotocin (STZ)-diabetic rats, an animal model of diabetic neuropathic pain (DNP).

This study aimed at evaluating the effects of TRPV1 activation at the RVM on pain modulation during DNP.

Male Wistar rats were rendered diabetic by an intraperitoneal injection of STZ. Control (CTRL) animals received the vehicle. A guide cannula was stereotaxically implanted at RVM in all animals. Formalin-induced nociceptive behaviors and mechanical nociception (paw pressure test – PPT), were evaluated in CTRL and STZ-diabetic rats, after capsaicin (TRPV1 agonist) or vehicle (DMSO) injections. Additionally, TRPV1 expression and endovanilloid levels were quantified in the RVM of STZ-diabetic rats with 4 weeks of diabetes and age-matched healthy control animals, by counting the number of TRPV1-immunopositive neurons and by mass spectrometry, respectively.

Administration of capsaicin induced a significant decrease in pain behaviors during the 2nd phase of formalin test in STZ-diabetic rats, but not in control animals. No effects of capsaicin were perceived during the 1st and quiescent phases of the formalin test. Capsaicin reversed the mechanical hypersensitivity detected in STZ-diabetic rats, with a maximum peak effect at 30 min post-administration. No effects were observed in mechanical nociception of CTRL rats.

An up-regulation of TRPV1 was observed in the RVM of STZ-diabetic rats, as these animals presented a significantly increased numbers of TRPV1-expressing neurons when compared with CTRL rats. A significant decrease in the levels of endovanilloids, namely anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), was also detected in the RVM of STZ-diabetic rats.

These results demonstrate that activation of TRPV1 potentiates RVM-mediated descending antinociception during DNP, but has no effects on pain modulation in healthy conditions. Diabetes-induced disruption in endovanilloid signaling may impair RVM-mediated descending pain modulatory mechanisms, subserving the diabetic neuropathic pain condition.

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Stg/Cdc25 phosphatase as a potential modulator of TAU neurotoxicity

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Tauopathies, including Alzheimer disease (AD) are characterised by the intracellular deposition of neurofibrillary tangles (NFTs), composed mainly by hyperphosphorylated forms of the microtubule-associated protein TAU. The molecular mechanisms that underlie Tau-induced neurotoxicity are still unclear. Thus, understanding the molecular and cellular pathways perturbed by Tau in disease states still remains a critical question and is essential for the development of disease therapies. *Drosophila melanogaster* has been used to model several human neurodegenerative diseases, including tauopathies. The fly amenability to perform genetic screens combined with its powerful genetics, and the finding that most human disease-associated genes have an evolutionary conserved fly homologue make it an ideal model to address basic questions associated with the onset of neurodegenerative phenotypes. We are using the fly visual system as a model to uncover the initial steps that may lead to neurodegeneration in tauopathies. *Drosophila* models of human tauopathy have been previously established[1,2]. When ectopically expressed during *Drosophila* eye development, hTau leads to a rough eye phenotype that can be used as readout of Tau neurotoxicity. The phosphatase encoded by *Stg/Cdc25 (String)*, the universal G2/M regulator, was identified as a strong suppressor of Tau- associated neurotoxicity. Yet, the molecular mechanisms that underlie this genetic interaction have never been addressed. Whether Stg activity is essential to modulate Tau phosphorylation levels and activity is still unknown. The aim of this project is to uncover the mechanisms that underlie Stg-Tau interaction, using a genetic and biochemical approach. We will present our latest findings on the potential role of *Stg/Cdc25* as a modulator of Tau neurotoxicity.

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Involvement of Neuropeptide Y system through Y_1 receptor in the resorptive capacity of osteoclasts *in vitro*

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Introdution: Local and systemic factors, including Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL) and Macrophage Colony-Stimulating Factor (M-CSF) regulate bone remodeling and metabolism. These two cytokines have an essential role in the formation and activation of osteoclasts (bone-resorbing cells) both *in vivo* and *in vitro* [1]. Our group has previously shown that Neuropeptide Y (NPY) system through Y_1 receptor (Y_1R) contributes for bone homeostasis via a direct action on osteoblasts (bone-forming cells) [2]. However, the direct role of this pathway in osteoclast activity remains to be clarified. The aim of this work is to analyse the effect of Y_1R blockade in the regulation of osteoclast function.

Materials and Methods: Osteoclasts were generated from mouse bone marrow by treating the cultures with 100 ng/mL RANKL, 30 ng/mL M-CSF and 60 nM Y_1R antagonist BIBP3226 for 21 days and the effect of BIBP3226 in osteoclast resorptive ability was analysed at the end of culture on dentine slices.

Results and Discussion: Our preliminary results demonstrate that osteoclast cultures treated with 60 nM Y_1R antagonist BIBP3226 show a large decrease of resorption area on dentine slices, compared to the control condition.

Herein, we present the first group of evidences suggesting that NPY system plays a role in the regulation of osteoclast function *in vitro* involving Y_1R.

To confirm this conclusion, we intend evaluate the resorptive capacity of osteoclasts derived from bone marrow of germline Y_1R knockout mice.

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Satellite Glial Cells surrounding primary afferent neurons become activated and proliferate during monoarthritis

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Satellite Glial Cells (SGCs) encircling primary afferents at the dorsal root ganglia (DRG) have been recently described as crucial players in the establishment/maintenance of chronic pain states. In fact, after peripheral injury or/and inflammation, SGCs are known to display activation and proliferation. It has also been suggested that the expression of neuronal injury factors might initially trigger these events in SGCs. We then aimed at evaluating the involvement of SGCs in the establishment/maintenance of an articular inflammation, by using the monoarthritis (MA) model. Moreover, we intended to investigate if the neuronal injury marker activating transcriptional factor 3 (ATF3) is associated with these SGCs' reactive changes, since we previously showed it is up-regulated in the early stages of MA.

Western Blot (WB) analysis of the glial fibrillary acidic protein (GFAP) expression was performed in L4-L5 DRGs from control non-inflamed rats and MA animals at different time-points of disease (4, 7 and 14d, induced by complete Freund's adjuvant injection into the left hind paw ankle joint). Data indicate that SGCs activation is occurring in MA animals, particularly after day 7 of disease evolution. Additionally, double-immunostaining for ATF3 and GFAP in L5 DRG sections shows that SGCs' activation significantly increases around stressed neurons at 7d of disease, when compared with control animals. The specific labelling of GFAP in SGCs rather than in other cell types was also confirmed by immunohistochemical labeling. Finally, BrdU incorporation indicates that proliferation of SGCs is also significantly increased after 7 days of MA.

Data indicate that SGCs play an important role in the mechanisms of articular inflammation, and suggest that ATF3 might be involved in SGCs' reactive changes such as activation. Further studies are still necessary to evaluate the possibility of SGCs and ATF3 constituting novel targets for therapeutic approaches.

Optimization of *in vitro* platforms for innervation/osteoclastogenesis cellular communication

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In the local bone microenvironment, several evidences suggest that peripheral innervation modulates bone resorption (Togari, 2002). However, the crosstalk between peripheral nerve terminals and osteoclasts, cells responsible for bone degradation, has not yet been clarified.

Therefore, the aim of this work is to optimize platforms, based on compartmentalized microfluidic devices, to obtain 2D and 3D enhanced coculture systems that better mimic the microenvironment of cellular communication between osteoclasts and sensory neurons.

Entire DRGs from adult origin were obtained from the excised spine of C57Bl6 mice (6-8 weeks old) and cultured in the somal side of microfluidic chambers as described in Neto *et al.* 2014. Bone marrow-derived osteoclasts were obtained from mice femur and tibia after differentiation with macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappaB ligand (RANKL) during three days. Afterwards, osteoclastic cells are detached and seeded in the axonal side of the microfluidic chambers.

Currently, we are optimizing the culture of osteoclasts in microfluidic chambers, providing enriched media and a mineralized matrix that can guarantee cell activation and mimic *in vivo* conditions. We started by optimizing the cell density and we observed that $3x10^4$ cells/channel provided the best result concerning cell adhesion and distribution within the microfluidic channel. Secondly, we increased the microfluidic area in the axonal side in view of getting a more homogeneous cell distribution, but we verified that most platforms failed to adhere to the glass coverslips. Subsequently, and in order to improve the osteoclasts substrate, we performed different coatings in the axonal side. Collagen and collagen-hydroxyapatite (HA) (30:70) were tested. We verified that, while collagen seemed to get slowly degraded throughout the first 3-5 culture days, collagen-HA was rapidly (hours) degraded and detached from the coverslips, probably due to the lack of a crosslinking agent. To overcome this problem, two different approaches, namely biomimetic deposition and spin coating, will be tested. Finally, we will investigate the effect of osteoclast soluble factors on axonal outgrowth.

Together, the obtained results will provide important knowledge to establish 2D and 3D neuronal-osteoclast cocultures using microfluidic technology.

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Antagonize without compromise: Effect of Y1 receptor antagonist on bone re-innervation

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Bone innervation is described to be a critical step to bone regeneration. During bone fracture healing there is a peak of re-innervation which, when compromised, results in a delay on fracture healing[1]. Nerve fibers present at the fracture site were observed to be immunoreactive to neuropeptide Y (NPY)[2] that is highly involved on the control of bone mass through Y_1 receptor (Y_1R) signaling[3]. Moreover, the systemic blockade of Y_1Rs by administrating orally an Y_1R antagonist led to a higher bone mass phenotype in mice[4], enforcing the importance of Y_1R in bone regeneration. Still, given the importance of re-innervation during bone regeneration/healing and the expression of Y_1R by sensory neurons, it remained to be explored the effects of antagonizing Y_1R on sensorial re-innervation.

Entire dorsal root ganglia were obtained from 7-week-old C57BL/6 male mice and plated in microfluidic chambers (Millipore) coated with 0.1 mg/ml PDL and 5 µg/ml laminin, using neurobasal medium. Different concentrations of Y_1R antagonist – BIBP3226 (Tocris, Bioscience) – were added to axonal side (6, 60, 600 and 1000 nM). Images were obtained at pre- and post-treatment time points. Time-lapse videos were captured during the first 8h of treatment. Immunocytochemistry against neuronal specific marker - βIII tubulin - were performed in order to quantify the axonal outgrowth using a developed custom-made algorithm (MATLAB, R2010a).

Microfluidic devices were used in order to evaluate the influence of BIBP3226 (Y_1R antagonist) in the axonal outgrowth. At first hours post-treatment (8h) it was observed that there was a neurite retraction upon the treatment with BIBP3226 when compared to control, by time-lapse live imaging.

After three days of treatment, images obtained from immunocytochemistry were used to quantify the axonal density spatial dependence using the algorithm developed with MATLAB. The algorithm was based on a moving column travelling across the longitudinal axis of the image giving the profile for each BIBP3226 concentration. Once more, a retraction in neurite outgrowth could also be detected. The observed effects were not dose-dependent.

This work provides new insights on the effect of a highly selective Y_1R antagonist (BIBP3226) in axonal outgrowth. In point of fact, our data demonstrates that all concentrations of BIBP3226 tested leads to a retraction in neurite outgrowth on sensory neurons. Envisioning a local therapy for bone regeneration, these findings are particularly relevant for the design of a drug delivery system based on Y_1R antagonism, in order to avoid interfering with the required nerve regeneration that leads to tissue healing/regeneration.

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Differential activation and transport of injury signals contributes to the failure of a dorsal root injury to increase the intrinsic growth capacity of DRG neurons

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Following peripheral nervous system injury, besides increased cAMP, the positive injury signals ERK, JNK and STAT-3 are locally activated and retrogradely transported to the cell body, where they induce a pro-regenerative program. Here, we used dorsal root ganglia (DRG) neurons which comprise a central branch that does not regenerate and a peripheral branch that regrows after lesion, to further understand the importance of injury signaling for successful axon regeneration. Although injury to the central branch of DRG neurons (dorsal root injury-DRI) activates the above positive injury signals and increases cAMP levels, it does not elicit the gain of intrinsic growth capacity nor the ability to overcome myelin inhibition, as occurs after injury to the peripheral branch (sciatic nerve injury- SNI). Besides, by blocking ERK activation and adenylyl cyclase activity, we show that the gain of intrinsic growth capacity of DRG neurons after injury is independent of ERK and cAMP. Antibody microarray analysis of axoplasm from rats with either DRI or SNI following dynein immunoprecipitation revealed a broad differential activation and transport of signals after each injury type and further supported that ERK, JNK, STAT-3 and cAMP signaling pathways are probably minor contributors to the differences in the intrinsic axon growth capacity observed in these injury models. From the injury signals differentially activated after DRI and SNI, we specifically identified increased levels of Hsp-40, ROCK-II and GSK3β after DRI, not only in axons but also in DRG cell bodies. In summary, our work shows that activation and transport of canonical positive injury signals is not sufficient to promote increased axonal growth capacity, and that a limited regenerative response after DRI may be accounted by the differential activation of inhibitory injury signals including ROCKII and GSK3β.

KIAA0319 is an inhibitor of axon growth acting through SMAD2 signaling

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KIAA0319, a transmembrane protein associated with dyslexia and neuronal migration, is highly expressed in DRG and motor neurons. Following injury, KIAA0319 expression is downregulated suggesting a regulatory role during axon growth. Supporting this hypothesis, overexpression of KIAA0319 in hippocampal neurons led to a specific decrease in axon growth, without affecting dendrite length or neurite formation. Using deletion constructs, the inhibitory KIAA0319 activity was mapped to the initial region of its cytoplasmic domain. By analysis of putative phosphosites and conserved regions present in this domain, KIAA0319 Y995A was identified as a dominant-negative mutant capable of reverting the inhibitory effect of KIAA0319. Neuronal overexpression of KIAA0319 specifically induced increased SMAD2 phosphorylation, which was reverted by overexpression of KIAA0319 Y995A. Moreover, KIAA0319 overexpression in the presence of a SMAD2 inhibitor reverted KIAA0319 activity, further supporting the central role of the SMAD2 pathway in the KIAA0319-induced repression of axon growth. We also observed decreased levels of tubulin (and no differences in actin levels) in the growth cones of KIAA0319 overexpressing neurons, suggesting that the microtubule organization is being affected. To understand the role of KIAA0319 in axon regeneration *in vivo*, mice with inducible neuronal deletion of KIAA0319 were generated. Following spinal cord injury, Thy1cre-KIAA0319$^{fl/fl}$ mice had a moderate increase in the number of regenerating axons. The modest effect observed with *in vivo* KIAA0319 deletion, suggested that high levels of KIA00319 expression, such as those observed in adult DRG and motor neurons, might restrict axon elongation rather than downregulation being capable of mediating a strong effect in axon growth. To further evaluate this hypothesis, we are currently establishing an *in vivo* overexpression model of KIAA0319 and identifying the intracellular KIAA0319 interacting partners that might regulate its function.

The homeodomain transcription factor Tlx3 directly promotes excitatory and suppresses inhibitory differentiation in spinal cord dorsal horn neurons

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The generation of excitatory and inhibitory neurons relies on combinatorial expression of transcription factors to induce specific neuronal differentiation programs. The *Tlx*-class homeobox genes Tlx3 and Tlx1 act as post-mitotic selector genes determining excitatory at the expenses of inhibitory neuronal fate in embryonic dorsal spinal cord (1). However, it is unknown whether *Tlx* genes act directly or indirectly to promote excitatory differentiation. Because *Tlx1* is expressed in a subset of *Tlx3*-expressing cells in cervical and thoracic spinal cord, where it partially compensates for the loss of Tlx3 function (1), we focused our analysis on Tlx3molecular mechanisms governing neuronal sub-type specification. Here we used chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) to map genome-wide Tlx3 binding sites in mouse embryonic dorsal spinal cord. Strikingly, we find that Tlx3 directly regulates distinct sets of transcription factors associated with excitatory or inhibitory cell fate specification. In addition, Tlx3 regulates terminal differentiation genes by directly promoting excitatory or repressing inhibitory gene markers' expression, supporting Tlx3 role as a master regulator in developing dorsal spinal cord. Moreover, Tlx3 directly binds Pax2 homeodomain factor providing a novel mechanism by which Tlx3 suppresses inhibitory differentiation.

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Involvement of pain facilitation areas of the brain in opioid-induced hyperalgesia

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Opiates are a gold standard for the treatment of moderate to severe pain. The chronic use of opioids is nonetheless also associated with paradoxical hyperalgesia (opioid-induced hyperalgesia; OIH). OIH is characterized by hypersensitivity to innocuous or noxious stimuli during sustained opiate administration but its molecular mechanisms are not fully understood. Several evidences indicate the contribution of increased pain facilitation. Here we studied the involvement of an area located in the medulla oblongata, the dorsal reticular nucleus (DRt), which plays a unique pain facilitatory role. We studied the effects of chronic morphine infusion in naïve and neuropathic animals (spared nerve injury-SNI-model) and evaluated the involvement of the DRt in OIH by pharmacological inactivation of the DRt and by knocking down the expression of the μ-opioid receptor (MOR) at the DRt.

Naïve and neuropathic male Wistar rats were anesthetized and implanted subcutaneously (s.c.) with osmotic pumps for morphine (45 $\mu g.\mu l^{-1}.h^{-1}$) or saline infusion. Pain behavior was tested 2, 4 and 7 days later by the von Frey and hot-plate tests in naïve animals and by the von-Frey, pin-prick and acetone tests in SNI animals. Pharmacological inactivation of the DRt was performed in naïve animals implanted in simultaneous with osmotic pumps s.c., for morphine or saline infusion, and stereotaxically with a guide cannula at the DRt. Lidocaine (0.5 μl; 4% w/v) was injected 7 days later, through the guide cannula and the animals were behaviorally tested by the von Frey and hot-plate tests before and 30 min after injection. MOR knock down was performed in naïve and neuropathic animals simultaneously implanted with osmotic pumps s.c., for morphine or saline infusion, and stereotaxically injected at the DRt with a lentiviral vector that knocks down MOR. Pain behavior was tested before and 7 days after surgeries as above. At the end of the experiments the animals were sacrificed by vascular perfusion and the brainstem was removed. MOR expression was evaluated by immunohistochemistry in 40 μm coronal sections encompassing the DRt.

Chronic morphine induced hyperalgesia both in naïve and SNI animals which was fully reversed by lidocaine. MOR expression was significantly higher in the morphine group both in naïve and SNI animals. MOR knock down prevented MOR up-regulation in the morphine group and decreased the expression of MOR in the saline group. MOR knock-down prevented the development of OIH in the morphine group and induced hyperalgesia in the saline group both in naïve and SNI animals.

Our results indicate that chronic morphine exposure induces OIH in naïve and neuropathic animals and, that the DRt is involved in the mediation of OIH, likely through MOR activation whose effects appear to switch from inhibitory to facilitatory upon chronic morphine treatment.

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Cytoskeleton remodeling as a target for TTR-induced Neurodegeneration

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Mutations in Transthyretin (TTR) are associated with familial amyloid polyneuropathy (FAP), a neurodegenerative disorder characterized by the deposition of insoluble TTR aggregates, particularly in the peripheral nervous system. However, the cellular and molecular mechanisms that underlie TTR toxicity in peripheral nerves are still unclear. This work aims at the elucidation of those mechanisms proposing cytoskeletal components as main targets for TTR-induced neurodegeneration.

Abnormalities in cytoskeletal organization are a common feature of many neurodegenerative disorders. Amyloidogenic proteins such as beta-amyloid peptide (Aβ) have been shown to mediate neurotoxicity by inducing major alterations in both microtubules and actin filaments. Thus, in this work, the cytoskeleton organization of cultures of dorsal root ganglion (DRG) neurons was analysed, treating the cells either with soluble TTR to mimic the physiological environment, or TTR oligomers to mimic the pathological conditions.

We observed that TTR oligomers promoted a complete reorganization of both actin and microtubules in the growth cones of DRG neurons, whereas soluble TTR had no significant effect. TTR oligomers led to a marked reduction in the growth cone area with a complete depletion of the typical filopodial and lamelipodial actin structures of neuronal growth cones. Regarding microtubule organization, in control neurons we observed the usual organization of neurons growing in permissive substrates with tightly bundled axonal microtubules entering the growth cone in half of the growth cones, whereas in the other half, microtubules splayed out as they entered the growth cone. In contrast, in neurons treated with TTR oligomers, while growth cones with splayed microtubules were still observed, the remaining half of the growth cones were dystrophic with microtubules confined to the central zone with little extension into the periphery.

Based on these observations, we progressed to the assessment of microtubule dynamics. Microtubule growth speed assays were performed as well as the evaluation of the post-translational modifications of tubulin by quantifying the ratio of acetylated (stable)/tyrosinated (dynamic) microtubules. TTR oligomers led to a decrease in the microtubule growth speed and an increase in the ratio of acetylated (stable)/tyrosinated (dynamic) microtubules, indicating a stabilization of microtubules in the presence of TTR oligomers. Currently, we are analysing the effect of TTR oligomers on axonal transport as well as on actin dynamics.

Taken in consideration the results obtained, our study proposes growth cone cytoskeletal components as a target for TTR-induced neurodegeneration in FAP.

Genetics of spinocerebellar ataxias in Portuguese families: screening for SCA15, SCA28 and SCA36

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The spinocerebellar ataxias are rare neurodegenerative diseases clinically and genetically very heterogeneous. To this moment, 32 autosomal dominant SCAs have been genetically identified and 24 causative genes implicated in these pathologies. Trinucleotide repeat expansions are the cause of nine SCAs (SCA1, 2, 3, 6, 7, 8, 12, 17 and DRPLA). Intronic pentanucleotide repeats, an ATTCT expanded tract or an insertion, are also causative of these pathologies (SCA10 and 31). Besides repeats, classical mutations are the origin of at least 12 of these diseases (SCA5, 11, 13, 14, 15, 18, 19/22, 23, 26, 27, 28, 35). More recently, intronic GGCCTG hexanucleotide expansions have been found in SCA36. Mutation screening in known SCA genes allowed us characterize approximately 200 Portuguese families. Machado-Joseph disease/SCA3 is the most frequent (52%) followed by DRPLA (5%) and SCA2 (2%), whereas the remaining showed very low frequencies or were not found. Thus, more than 100 Portuguese SCA families remain without a molecular diagnosis. Here we present the results of mutation screenings for SCA15, SCA28 and the recently described SCA36 in these Portuguese families. All previously reported SCA15 patients have deletions of several exons of the *ITPR1* gene; SCA28 is caused by missense mutations or small in/dels in the *AFG3L2* gene; GGCCTG hexanucleotide expansions, ranging from 650-2500 repeats, in intron 1 of *NOP56*, cause SCA36, the most common type of SCA in Galicia, the Spanish region on the border with Northern Portugal. We carried out quantitative real-time PCR, direct sequencing of exons of interest or repeated-primed PCR to screen for *ITPR1* genomic deletions, *AFG3L2* mutations and *NOP56* expansions, respectively, and failed to find pathogenic alterations. In conclusion, SCA15, SCA28 and SCA36 are very rare among Portuguese SCA families and most probably a considerable number of SCA genotypes remain to be identified.

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Refinement and experimental design in publications: a longitudinal case study of ALS research

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Amyotrophic Lateral Sclerosis (ALS) is a human neurodegenerative disease of rapid progression for which no effective treatment exists, resulting in progressive paralysis and death. Similar clinical signs cause distress to research animals modelling the disease. From a 3Rs perspective it is desirable to implement refinement measures such as humane endpoints and housing adaptations.

To evaluate whether measures for refinement and experimental design optimization were reported, published studies on murine ALS models were analyzed (n=267). Studies were classified according to a scale of severity, based on the disease stage that animals reached. Studies published in 2005, 2009 and 2011 were included to cover the period in which guidelines to improve preclinical ALS research were introduced[2, 3].

Report of compliance with regulation for animal use and care increased over the years reviewed (p<0.01), however the severity level remained unchanged (62% of studies implemented a late stage endpoint of disease progression). Preclinical studies were reported with significantly higher level of detail than proof-of-concept studies throughout the years. Compared with Huntington's disease[1], a similar neurodegenerative disease model for which no specific research guidelines exist, ALS research used less severe endpoints with humane endpoints reported in 90% of publications.

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In vitro biological evaluation of fibrin hydrogels functionalized with HYD1 and A5G81 peptides for transplantation of ES-derived neural stem cells into the spinal cord

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Introduction: Neural stem cell (NSC) transplantation is one of the most promising therapeutic approaches under investigation for the treatment of spinal cord (SC) injuries. Nevertheless, NSCs simply injected into the SC often show poor cell survival and limited cell migration. We propose to develop a fibrin (Fb)-based hydrogel for the delivery of NSCs into the injured SC. Fb will be functionalized with ligands for selective binding to α6β1 integrin receptors, an integrin highly expressed by NSCs that has been shown to modulate NSC migration.[1]

In this study, six synthetic peptides with reported high affinity to α6β1 integrin were investigated. The ones promoting the highest levels of cell adhesion/migration of NSCs mediated by α6β1 integrin were subsequently immobilized in Fb gel, and the ability of functionalized Fb to promote NSC migration subsequently assessed. Finally, the effect of immobilized α6β1 ligands on neurite extension was examined.

Materials and Methods: NSCs were derived from the ES-46C cell line in serum-free medium and adherent monoculture and seeded on peptide-adsorbed surfaces at different coating concentrations. Cell adhesion (in the presence/absence of mAbs against α6 and β1 integrin subunits), attachment strength, spreading, migration and differentiation along the neuronal lineage were quantitatively assessed. Bi-domain peptides with the selected α6β1 ligands at the carboxyl terminus and the factor XIIIa substrate domain at the amino terminus were bound to Fb gels at different concentrations (10 to 320 μM) by the action of factor XIIIa.[2] The amount of covalently bound ligands and their stability over time was determined using ^{125}I-labeled peptides. To assess NSC migration, neurospheres (NSC aggregates) were seeded on functionalized Fb gels and radial outgrowth determined after 72 h of cell culture. Unmodified Fb and Fb containing laminin 111 or recombinant human laminin 511 were used as controls. The effect of functionalized Fb gels on neurite extension was assessed quantifying neurite outgrowth from rat E18 dorsal root ganglia (DRG) cultured for 48 h in the modified gels.

Results: Among the tested peptides HYD1 and A5G81 were the most effective in terms of ability to promote cell adhesion and migration of NSCs, and selectivity towards α6β1 integrin.

Radiolabeling experiments revealed for input peptide concentrations ≤ 20 μM similar amounts of immobilized HYD1/ A5G81 in Fb (peptide binding efficiency of about 20%). Immobilized HYD1 was effective in promoting NSC migration, functionalization of Fb with 20 μM of bi-domain peptide leading to a 2.4-fold increase in NS outgrowth area. Neither immobilization of A5G81 nor the incorporation of soluble LN were able to promote NSC migration on Fb. Immobilized HYD1 showed to be efficient in promoting neurite extension from DRGs, independently of the concentration tested.

Discussion and Conclusions: Taken together these results point out that functionalization of Fb gel with HYD1, apart from promoting NSC migration, also enhances neurite extension of DRG neurons. Currently, HYD1-functionalized gels are being evaluated in terms of ability to promote NS outgrowth mediated by α6β1 integrin.

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Exploring PEG-GATG dendritic block copolymers as siRNA carriers

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Introduction: Among the approaches explored in gene therapy, the down-regulation of protein expression mediated by siRNAs is one of the most promising. As in the DNA case, its success is intimately related with the carrier's efficiency to encapsulate and deliver siRNA into target cells with minimal toxicity. Among the carriers, dendrimers are promising candidates due to their globular, branched and multifunctionalizable structure, monodispersity and their capacity to complex and protect DNA and RNA in compact structures.

However, the usual polymers proposed for DNA delivery are not necessarily optimal for siRNA, due to fundamental differences between both molecules such as size, morphology, flexibility and charge.

Recently, gallic acid-triethylene glycol (GATG) dendrimers and their PEG-dendritic block copolymers (PEG-GATG) have been proposed as pDNA vectors showing promising results.[1,2] Yet, our preliminary studies showed that they are not able to efficiently complex and protect siRNA.

Here we report that the surface modification of PEG-GATG dendrimers with different positively charged amine groups improves their capacity to encapsulate siRNA and so deliver it into cells.

Methods: PEG-GATG were functionalized at the peripheral azides with a diamine (D) and an aromatic amine (Ar) by click chemistry ($CuSO_4$, sodium ascorbate). The resulting products (PEG-GATG-D and PEG-GATG-Ar) were purified by ultrafiltration and characterized by NMR and MALDI-TOF.

PEG-GATG/siRNA dendriplexes with N/P ratios (moles of protonated amines/moles of phosphate groups) ranging from 20 to 160 were formed and characterized in terms of polydispersity (PDI), size and morphology by DLS and TEM. The complexation ability was studied by electrophoresis.

Dendriplex internalization was tested by imaging flow cytometry 24 h post-transfection using U2OS cells. The silencing efficiency studies, using GFP and Luciferase as reporter genes, are ongoing.

Results and discussion: PEG-GATG surface was successfully functionalized with two amine groups by click chemistry (Fig 1). PEG-GATG/siRNA dendriplexes showed sizes of 140 and 170 nm for PEG-GATG-D and PEG-GATG-Ar, respectively. The smaller PDI's observed for the PEG-GATG-Ar/siRNA particles indicate a higher homogeneity due to the additional hydrophobic interactions between the amine's aromatic ring and siRNA. Electrophoresis studies showed that PEG-GATG-D have higher complexation efficiency due to the multivalency of the diamine. TEM studies showed that both dendrimers complex siRNA in well-defined globular structures. The dendriplexes with higher N/P's were more efficiently internalized.

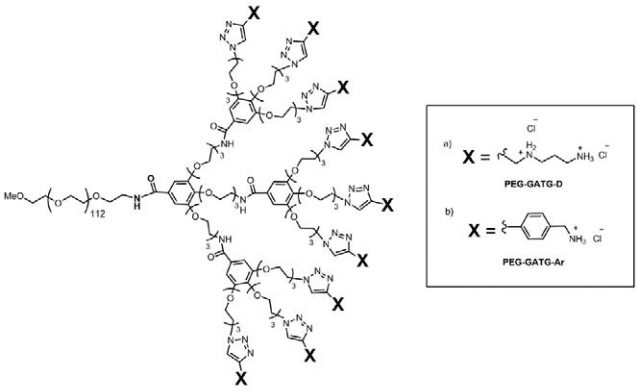

FIGURE 1: PEG-GATG-D and PEG-GATG-Ar dendrimers

Conclusions:

- Click chemistry together with ultrafiltration allowed the quick, efficient and reliable multivalent conjugation of unprotected amines to PEG-GATG dendrimers.
- Both PEG-GATG/siRNA dendriplexes showed suitable sizes and PDI's for cellular uptake.

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In vitro tools to explore the communication between injured bone and the hypothalamic Neuropeptide Y system

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Nervous system is recognized as a key regulator of bone homeostasis. Among the different pathways of bone regulation by the nervous system, the Neuropeptide Y (NPY) neuronal pathway has been attracting particular attention [1][2]. Recently, we showed that the hypothalamic NPY system responds to bone injury in mice. However, the processes of feedback from injured bone to the NPY neuronal pathway remain unknown. We hypothesize that systemic factors released after bone injury may cross and /or interact with the blood brain barrier (BBB) and contribute to the modulation of the NPYergic hypothalamic response. To address this hypothesis, we aimed to establish two crucial tools for an *in vitro* approach: hypothalamic organotypic cultures and *in vitro* BBB cultures.

The hypothalamic organotypic cultures were prepared from 8-days-old C57Bl/6 mice brains, based on the air-medium interface culture method [3]. We were able to achieve high cell viability (82 ± 9.6 %) and a dense neuronal network was observed. Furthermore, the stimulation with dexamethasone resulted in an increased NPY expression, confirming the responsiveness of the NPY system of these organotypic cultures.

The *in vitro* model of the BBB was performed according to a coculture system using a brain microvascular endothelial cell line (bEnd.3) and primary astrocytes [4]. The expression of junctional molecules as claudin-5, ZO-1, occludin and VE-cadherin was observed in the bEnd.3 cell-cell contact, confirming the BBB phenotype of these endothelial cells. Moreover, the transendothelial electrical resistance (TEER) values and the permeability coefficients (Pe) obtained in the transendothelial flux test support high integrity of the established barrier.

The successful establishment of hypothalamic organotypic cultures and an *in vitro* BBB model allowed to set-up an experimental platform to address the role of the humoral pathway in the feedback from injured bone to the CNS, specifically to the hypothalamic NPY system. The BBB cultures will be treated with serum collected from femur-defect animals, and the effects of the resulting conditioned medium on the NPY system activity in the hypothalamic organotypic cultures will be assessed. Factors able to cross and/or interact with the BBB and subsequently modulate the NPY system will be identified.

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Satellite Glial Cell Activation Occurs in the Collagenase Model of Osteoarthritis in Rats

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Background and aims: Pain is a major feature of osteoarthritis (OA), but its therapy is still highly ineffective. Recently, the overexpression of glial fibrillary acidic protein (GFAP), a known marker of satellite glial cell (SGC) activation, has been reported in several animal models of pain. Here, we evaluated if this is also observed in the dorsal root ganglia (DRG) of rats with OA induced by intra-articular injection of collagenase, hoping to contribute to a better understanding of the pain mechanisms in this pathology.

Methods: OA was induced by double injection of 500U of type II collagenase into the knee joint of rats, at days 0 and 3. Movement-induced nociception was evaluated weekly, until sacrifice, by the Knee-Bend and CatWalk tests. Animals were sacrificed 6 weeks after the first collagenase injection (n=6/group). L3-L5 DRGs were used for immunodetection of GFAP, and knee joints were processed for histopathological evaluation.

Results: Collagenase injected into the knee joint induced an increase in movement-induced nociception when evaluated by both the Knee-Bend and Catwalk tests. Histopathological changes observed after 6 weeks of OA induction were very similar to those described in clinical OA, leading to extensive cartilage degeneration with fissures and exposure of the subchondral bone. GFAP expression in the SGC of collagenase-injected animals was significantly increased when compared to control animals.

Conclusion: The intra-articular injection of collagenase leads to SGC activation, which may be implicated in the development of chronic pain in this model.

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Activation of dopaminergic D2/D3 receptors reverses hippocampus-related working memory deficit induced by neuropathic pain

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Dopamine plays an important role in several forms of synaptic plasticity in the hippocampus, a crucial brain structure for working memory (WM) functioning. In this study, we evaluated whether the working-memory impairment characteristic of animal models of chronic pain is dependent on hippocampal dopaminergic signaling. To address this issue, we implanted multichannel arrays of electrodes in the dorsal and ventral hippocampal CA1 region of rats and recorded the neuronal activity during a food-reinforced spatial WM task of trajectory alternation. Within-subject behavioral performance and patterns of dorsoventral neuronal activity were assessed before and after the onset of persistent neuropathic pain using the Spared Nerve Injury (SNI) model of neuropathic pain. Our results show that the peripheral nerve lesion caused a disruption in WM and in hippocampus spike activity and that this disruption was reversed by the systemic administration of the dopamine D2/D3 receptor agonist quinpirole (0.05 mg/kg). In SNI animals, the administration of quinpirole restored both the performance-related and the task-related spike activity to the normal range characteristic of naive animals, whereas quinpirole in sham animals caused the opposite effect. Quinpirole also reversed the abnormally low levels of hippocampus dorsoventral connectivity and phase coherence. Together with our finding of changes in gene expression of dopamine receptors and modulators after the onset of the nerve injury model, these results suggest that disruption of the dopaminergic balance in the hippocampus may be crucial for the clinical neurological and cognitive deficits observed in patients with painful syndromes.

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JMY, a new player in oligodendrocyte process extension and early axon-glia interaction

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The formation of myelin sheath around axons in the central nervous system is carried out by oligodendrocytes (OL). The ensheathment and wrapping steps of myelination are extremely complex and highly dynamic events that require a rearrangement of the cytoskeleton . Although much remains to be learned about this process, it is clear that actin-related proteins are important players and regulators of these events. We are interested in further characterizing the actin-regulating protein network that dictates how OLs extend processes, interact with the axon and starts wrapping around it. We found that a novel protein, JMY, also known as junction-mediating and regulatory protein p53 cofactor, is upregulated in the processes of OLs, when compared with its expression in the soma, during the initial phase of process extension. JMY has been described as a dual functional protein that can act as an actin nucleator when in the cytoplasm, through Arp2/3 activation or trough direct actin nucleation by itself, and can also be a p53 cofactor when in the nucleus. Therefore, we asked how Jmy is modulating process extension and early axon-glia interaction and if it has a function in axon ensheathment and wrapping. Using primary cultures of rat oligodendrocyte progenitor cells, we observed that JMY is present during OL differentiation *in vitro*, with maximal expression in fully differentiated, mature OLs. Furthermore, we analyzed its expression in mouse tissues from central and peripheral nervous system and saw that JMY is present in the optic nerve and in the sciatic nerve at different developmental timepoints of myelination. Knockingdown Jmy in OLs *in vitro* leads to dramatic morphological defects, including fewer and shorter processes with reduced branching. However, depletion of Jmy does not seem to impact the expression of myelin genes, such as MBP (myelin basic protein). This presents as a very interesting example to dissect how ubiquitous molecules differentially regulate form and function, especially in a cell where the two are intrinsically linked. We are now using a co-culture subsystem of dorsal root ganglion (DRGs) and oligodendrocytes lacking Jmy to assess whether Jmy is required for normal axon-glia early interaction. In the future, we plan to perform live cell imaging of these co-cultures to get insight on how this dynamic process takes place and the OL cell shape impacts the formation of the myelin sheath around an axon.

Farber disease: a fatal childhood disorder of sphingolipid metabolism

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Farber Disease, also known as Farber Lipogranulomatosis (MIM 228000), is a rare recessively inherited disorder characterized by intralysosomal accumulation of ceramide due to the deficiency of acid ceramidase. Typical symptoms, generally noticed early in life, include the unique triad of subcutaneous nodules, painful and progressively deformed joints, and hoarseness by laryngeal involvement. Based on the age of onset, the severity of symptoms, and the extension of ceramide storage seven disease subtypes are distinguished. Subtypes 1 to 6 are associated with the enzymatic deficiency of acid ceramidase caused by mutations in the *ASAH1* gene, whereas subtype 7 is caused by the deficiency of the sphingolipid activator protein precursor. Comparing with other sphingolipidoses, a small number of Farber patients have been identified worldwide, thus suggesting that the disease may be globally underestimated. Indeed, as far as we know no Portuguese Farber patient has been identified so far. With the aim to increase the awareness of Farber Disease and help in the identification of potential Farber cases, information on this disease has been disseminated to Portuguese physicians. In this presentation, the clinical similarities with other diseases will be firstly underlined. The importance of patient genotype ascertainment will be then highlighted since this activity not only will improve the accuracy of the disease diagnosis but also foster the comprehension on the disease pathogenesis, thus being of crucial value for future therapeutic interventions. All interested in this disease and willing to contribute to the knowledge of the genetic epidemiology of Farber disease are invited to collaborate with us.

Dissecting the role of cytoskeleton remodeling in transthyretin-induced neurodegeneration in a *Drosophila* model

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Transthyretin (TTR) is a protein mainly synthesized by the liver and the choroid plexus of the brain, whose mutations are associated with familial amyloid polyneuropathy (FAP) [1]. FAP is characterized by the deposition of mutated TTR, in the form of amyloid fibrils, particularly in the peripheral nervous system (PNS) [2]. The most common pathogenic substitution is Val30Met and as a consequence of TTR deposition there is axonal degeneration that results in neuronal dead with disease progre ssion [2].

Drosophila as a model is impressively fast and particularly amenable to genetic manipulations and unbiased genetic screens. *Drosophila* research has been a source for new concepts in neurobiology, and work with fly revealed that cellular mechanisms have turned out to be conserved in higher animals. Fly neurons offer a number of useful features to study the neuronal cytoskeleton machinery, since in this model actin and microtubule binding proteins display high homology with mammalian ones [3].

In order to determine *in vivo* if cytoskeleton remodeling is a target for TTR-induced neurodegeneration, we will use the FAP *Drosophila* model (GMR-Gal4;UAS-TTR V30M) [4]. This fly model expresses TTR V30M in all cells of the developing eye including photoreceptors (R cells), leading to fibrillar deposition, which results in roughening of the eye and degeneration.

We will start by analyzing the cytoskeleton organization of R cells *in vivo* at different development stages and determine whether a defect in the axonal cytoskeleton precedes neurodegeneration. Alternatively, to access high resolution at subcellular level we will proceed with *Drosophila* primary neuronal cultures and investigate cytoskeleton changes. Moreover, we will conduct a genetic screen to identify enhancers and suppressors of TTR V30M-induced rough eye phenotype in order to clarify the mechanism related with neuronal cytoskeleton defects. Flies expressing TTRV30M will be crossed with readily available fly lines for knockdown (UAS-RNAi) or overexpression (UAS-gene) of candidate genes whose function is associated with cytoskeleton dynamics. Among these are Rho GTPases, motor proteins, GSK3, Cdk5, Phosphatases, microtubule and actin binding proteins, and tubulin acetylases/deacetylases.

In conclusion, this work will clarify whether cytoskeleton defects underlie TTR induced-neurodegeneration and provide the respective downstream targets.

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Development and biological characterization of a neuron specific non-viral delivery system for anti-Rho-kinase 1 and 2 short interfering RNA (siRNA-ROCK1/2)

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Introduction. Spinal cord injury (SCI) is a devastating condition leading to persistent dysfunction without recovery. Injured axons are prevented from regrowth and re-establish synapses by neurite outgrowth inhibitors present in scar tissue and myelin. These bind to specific receptors located in neurites, leading to activation of the Rho–ROCK pathway. As a result, neuronal growth-cone collapses causing the retraction of neurites and regeneration impairment[1].

Here we describe the development and characterization of a neuron specific non-viral delivery system for anti-Rho-kinase 1 and 2 short interfering RNA (siRNA-ROCK1/2). *N,N,N*-trimethyl chitosan (TMC) was used, as this polymer proved to mediate efficiently siRNA delivery to neuronal cells[2].

To confer neuron-specificity, the TMC/siRNA-ROCK1/2 polyplexes will be functionalized with the non-toxic carboxylic terminal fragment from the tetanus toxin (HC)[3].

Methods. TMC with a molecular weight of 43.3 kDa, degree of acetylation of 11.1% and degree of quaternization of 30% was initially modified with 2-iminothiolane to allow the binding of neuron-specific moieties. The resulting TMCSH was used to prepare polyplexes with different polymer to siRNA ratios (expressed as *N/P* ratios, where N is the moles of cationic nitrogens in the TMCSH and P is moles of phosphates in siRNA). TMCSH polyplexes from N/P 1 to 80 were prepared by adding a siRNA solution to a TMCSH solution (in 20 mM HEPES/5% Glucose and 50 mM Na_2SO_4) under mild vortex followed 10 min complexation at 1000 rpm and a 24h incubation period at RT. Polyplexes were then characterized in terms of size and polidispersity index (dynamic light scattering - DLS) as well as in terms of complexation capacity (gel retardation assay). TMCSH/siRNA complex stability in the presence of serum was also assessed.

Results. TMC was successfully grafted with 5% thiol groups per free amine. TMCSH is able to complex with siRNA-ROCK. Varying the *N/P* ratio resulted in different nanoparticle sizes (diameter) and polydispersity indexes. *N/P* ratios equal or higher than 5:1 led to particles with sizes ranging from 184 to 223 nm and polydispersity indexes from 0.26 to 0.5. When analysed in the presence of serum TMCSH/siRNA-ROCK polyplexes remained stable, showing siRNA complexation rates near 100%.

Presently we are evaluating these complexes in terms of charge, as well as internalization and transfection efficiency using neuronal cells.

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GABA-mediated pronociception of spinal 5HT3 receptor during painful diabetic neuropathy

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Spinal 5HT3R-dependent descending pain facilitation has recently been implicated in the development of inflammatory and traumatic neuropathic pain. Nevertheless, the contributions of 5HT3R to diabetic neuropathic pain (DNP), as well as the mechanisms underlying 5-HT3R-mediated spinal pain modulation remain elusive. 5HT3R activation enhances spinal GABA release which, in normal situations, inhibits spinal pain neurotransmission. However, DNP is accompanied by a shift in spinal GABA role, which was shown to exert excitatory instead of inhibitory effects. In this context, the GABA release induced by 5HT3R activation is likely to exert pro-nociceptive effects, contributing to spinal 5-HT3R-mediated pain facilitation. This study aimed to evaluate the contribution of spinal 5HT3R to diabetic neuropathic pain facilitation and to evaluate the GABA-dependence of 5-HT3R-induced pronociception.

Male Wistar rats were rendered diabetic by an intraperitoneal injection of STZ. Control (CTRL) animals received the vehicle. A catheter was implanted in the lumbar subarachnoid space and at 4 weeks post-injection, mechanical nociception was evaluated by paw-pressure test (PPT) in CTRL and STZ rats, before and at 0.5, 2, 4, 6, 24h after intrathecal (i.t) administration of saline/Y25130 (5-HT3R specific antagonist). Additional experimental groups were performed to evaluate the spinal nociceptive activation (ERKs pathway activation) and the spinal expression of 5-HT3R at the peak maximum effect elicited by Y25130 using immunohistochemistry and Western blotting techniques. In order to evaluate if 5-HT3R-mediated pronociception depends on GABA neurotransmission, additional saline/Y25310-treated STZ animals received an i.t injection of muscimol (GABAAR agonist), 30 min prior to the peak maximum effect of Y25310, and mechanical nociception was evaluated 30 min later.

STZ rats developed mechanical hyperalgesia as demonstrated by the significantly lower pre-treatment PPT values when compared with CTRL animals. Infusion of Y25130 significantly increased the PPT values of STZ rats, with a maximum peak effect at 4h post-administration, but had no effect in mechanical nociception of CTRL rats.

Immunohistochemical/western blot studies revealed that 5HT3R antagonist attenuated STZ-induced spinal hyperactivity, as demonstrated by decreased spinal ERK activation. Spinal 5HT3RA expression is unaltered by diabetic neuropathic condition. The administration of muscimol abolished the antinociceptive effect observed in Y25130-treated STZ rats and had no effects in saline-treated animals.

These results demonstrate that activation of spinal 5-HT3R facilitates pain transmission during DNP and that this effect is likely to result from the increased spinal GABAergic neurotransmission triggered by this receptor activation. The use of 5-HT3R antagonists may then be considered as a promising pharmacological treatment in alleviating diabetes-induced painful symptoms.

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Biomaterials with radical oxigen species scavenger capacity as a therapeutic strategy to manage age related macular degeneration

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Age related macular degeneration (AMD) is the leading cause of blindness in the elderly worldwide. A progressive loss of central vision occurs that can be attributable to degenerative and neovascular changes in the macula.[1] It is hypothesized that by modulating the ROS synthesis/degradation balance one counteract the processes, which contribute to systemic and tissue ageing of the eye and so the progression of AMD.[2-4] The ultimate objective of this project is to develop a novel class of hybrid biomaterials based on TiO_2 with integrated bioactivity in terms of ROS scavenging. There are three main crystalline phases of TiO_2, anatase, rutile and brookite, which differ in their physicochemical behaviour. Also, different calcination temperatures will lead to distinct particle sizes and surface area. Taking this into consideration, here we describe the effect of TiO_2 powders with different characteristics in the scavenging of radical species generated chemically or biologically. Also, the cytotoxicity effect of these materials was assessed either with inflammatory cells or cells present in the blood-retinal barrier (BRB).

Firstly, we assessed the ROS scavenging capacity of different TiO_2 powders with chemical assays using artificial ROS. The results indicate that the 200ºC and 600ºC calcined TiO_2 powders promote the best reduction of ROS concentration. Next, we assessed the bioactivity of these powders in the presence of macrophages. A viability assay showed that the powders have no toxic effect in macrophages at concentrations of 5 mg/ml or below, and do not promote macrophage activation. Also, we observed that the powder with the best scavenging capacity was the 600ºC calcined TiO_2 powder, despite having the lowest specific surface area, leading to 30% reduction in the ROS detected inside macrophages when applied at 1 mg/ml. At present we are extending the work to RPE cells, components of the BRB. We performed a viability assay and observed that the powders have no toxic effect on RPE cells at concentrations of 0,1mg/ml or below. The effect of the powders is being assessed on RPE cells kept under oxidative stress conditions caused by the presence of ROS, induced by the two oxidants.

Taken together, these results suggest that the TiO_2 powders with the best ROS scavenging capacity are the 200ºC and 600ºC calcined powders, showing no toxic effect on cells at lower concentrations. The produced TiO_2 powders, being materials capable of ROS scavenging either of artificial ROS or ROS generated by inflammatory cells, present themselves as valuable tools in the design of new strategies for application in the treatment of AMD.

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Evolution of eye and head size and shape between *Drosophila novamexicana* and *Drosophila americana*

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One major aim of biological research is to understand the processes involved in the origin of the amazing diversity of life forms on earth. Changes in embryonic and postembryonic developmental programs are responsible for the diversification of adult morphology and research in the field of evolutionary-developmental biology (evo-devo) aims to reveal the developmental basis for this variation.

The insect compound eye facilitates fundamental processes like feeding and reproduction, since it perceives light and is essential for integrating environmental information. Thus, changes in eye morphology within and between species should be accompanied by functional adaptations to an ever changing environment. A detailed analysis of three species of the *D. melanogaster* subgroup revealed that *D. mauritiana* eyes are significantly larger than those of its sibling species *D. melanogaster* and *D. simulans* due to facet size, while intraspecific differences in eye size are due to changes in ommatidia number. Thus, it is imperative to study divergent groups of species to determine if changes in eye size are governed by general or specific mechanisms across the entire *Drosophila* genus.

Preliminary data shows major differences in eye size between species of the *D. virilis* group which are largest between *D. novamexicana* (smallest eyes) and *D. americana* from the south of the distribution (biggest eyes). These species are diverging from *D. melanogaster* for at least 40 million years, and therefore, represent an excellent model to find out if natural variation in eye size and head morphology is due to the same changes in the underlying gene regulatory network in divergent *Drosophila* lineages. In order to reveal the major genomic regions responsible for the observed differences in eye size and head morphology, a genotype-phenotype association study involving *D. novamexicana* and *D. americana* from the south of the distribution is being performed.

Effect of TTR in Aβ uptake and degradation by the liver

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Transthyretin (TTR) is destabilized in Alzheimer's disease (AD) affecting its binding to Aβ and protection against noxious effects. We propose the use of small compounds known to stabilize the TTR tetramer fold thereby improving TTR/Aβ binding. Administration of one of such drugs – iododiflunisal - to a mouse model of AD resulted in reduced plasma Aβ levels thus suggesting that TTR participates in Aβ clearance at the periphery. The specific mechanism by which Aβ is cleared remains to be clarified. It is known that the Aβ bound to ApoE/cholesterol can be incorporated in HDL to be further delivered to the liver for degradation. The rapid peripheral clearance of Aβ is mediated mainly by hepatic LRP1, and this is blocked by RAP. TTR, which is mainly synthesized by the liver, also suffers hepatic degradation and its receptor, although never unequivocally identified, has been characterized as a RAP-sensitive receptor. Curiously, a fraction of TTR is transported in HDL, thus it is possible that TTR participates in Aβ clearance through the LRP receptor, at the liver.

Here we aimed at investigating the role of TTR in Aβ liver uptake and degradation, using cellular systems both a cell line (SAhep) and primary cultures of hepatocytes derived from mice with different TTR genetic backgrounds. Our results indicated that human recombinant TTR increases Aβ internalization in SAhep cells, as determined by flow cytometry analysis. We were also to able to optimize the isolation and establishment of primary cultures of hepatocytes derived from mice with two TTR gene copies ($TTR^{+/+}$), only with one copy ($TTR^{+/-}$) or without TTR ($TTR^{-/-}$), that will be key in confirming the importance of TTR in Aβ elimination by the liver.

Interleukin-1 signaling pathway as a therapeutic target in transthyretin amyloidosis

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Inflammation is a key pathological hallmark of several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and familial amyloidotic polyneuropathy (FAP). Among all inflammatory cytokines associated with FAP, interleukin-1β (IL-1β), in particular, has been implicated in playing a key pathogenic role. In the present study, we sought to investigate whether blocking IL-1β signaling provides disease-modifying benefits in an FAP mouse model. We assessed the effect of chronic administration of Anakinra, an IL-1 antagonist, on FAP pathogenesis *in vivo*, using real-time polymerase chain reaction, semi-quantitative immunohistochemistry, western blot and nerve morphometric analyses.

We found that treatment with Anakinra prevents transthyretin (TTR) extracellular deposition in sciatic nerve, protecting unmyelinated nerve fibers from aggregate-induced degeneration. Moreover, Anakinra administration significantly suppressed IL-1 signaling pathway and inhibited apoptosis and nitrative stress.

The present work highlights the relevance of the IL-1 signaling pathway in the pathophysiology of FAP. Our results bring to light the importance of non-amyloid targets in the therapeutic strategies for this disorder. Thus, we propose the use of Anakinra as a potential therapeutic agent for TTR-related amyloidosis.

Transthyretin chemical chaperoning by flavonoids: structure-activity insights towards the design of optimized pharmacological agents for transthyretin amyloidosis

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Transthyretin (TTR) variants with decreased conformational stability are related with different forms of hereditary amyloidoses and in particular with Familial Amyloidotic Polyneuropathy (FAP). Through a combination of compound library screens and epidemiological data, many small compounds, including natural occurring flavonoids, have been proposed has broad-spectrum inhibitors of amyloid formation. We have previously shown that (-) epigallocatechin-gallate (EGCG), the major catechin in green tea, strongly impairs transthyretin (TTR) aggregation and toxicity *in vitro* (Ferreira *et al.*, FEBS Lett., 2009, 2011). In addition, sub-chronic supplementation of FAP mice model with EGCG significantly decreased TTR burden and several biomarkers associated with non-fibrillar TTR deposition, namely endoplasmic reticulum-stress, protein oxidation and apoptosis markers along the gastrointestinal tract and peripheral nervous system (Ferreira *et al*, PLoS ONE, 2012). Our observations have been supported by an observational study on the effects of green-tea extract (GTE) consumption in patients with TTR cardiomyopathy showing an inhibitory effect of GTE on the progression of cardiac amyloidosis (Kristen *et al.*, Clin. Res. Cardiol., 2012). Nonetheless, the precise molecular details involved in flavonoid, in particular EGCG, interaction with TTR amyloidogenic intermediaries are still elusive and remain to be further explored.

To investigate the structure-activity relationships of plant flavonoids, we compare the effect of structurally related catechins and their larger polymers theaflavins, side-by-side, on TTR amyloid formation process by different biochemical and biophysical approaches, including dynamic light scattering, transmission electron microscopy, isoelectric focusing, cell culture and cell toxicity assays. Comparison of the effects of flavonoids on inhibition of TTR aggregation and toxicity appears to correlate closely with i) the presence of gallate ester moiety in the catechin structure and ii) the number of hydroxyl groups in the B-ring catechin structure.

On basis of the structure-activity studies presented here, we highlight the galloyl moiety as the key structural feature in chemical chaperoning of TTR abnormal self-assembly process by flavonoids and contribute to further unveil their mechanism of action as amyloid inhibitors. Furthermore, our findings may drive the design of optimized disease-modifying pharmacological agents for amyloid-related neurodegenerative diseases.

Molecular tweezers targeting transthyretin amyloidosis: trapping transthyretin misfolding and aggregation

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Transthyretin (TTR) amyloidoses comprise a wide spectrum of acquired and hereditary diseases triggered by extracellular deposition of toxic TTR aggregates in various organs. Despite recent advances regarding the elucidation of the molecular mechanisms underlying TTR misfolding and pathogenic self-assembly, there is still no effective therapy for treatment of these fatal disorders. Recently, the "molecular tweezers", CLR01, has been reported to inhibit self-assembly and toxicity of different amyloidogenic proteins *in vitro*, including TTR, by interfering with hydrophobic and electrostatic interactions known to play an important role in the aggregation process. In addition, CLR01 showed therapeutic effects in animal models of Alzheimer's disease and Parkinson's disease.

Here, we assessed the ability of CLR01 to modulate TTR misfolding and aggregation in cell culture and in an animal model. In cell culture assays we found that CLR01 inhibited TTR oligomerization in the conditioned medium and alleviated TTR-induced neurotoxicity by redirecting TTR aggregation into the formation of innocuous assemblies. To determine whether CLR01 was effective *in vivo*, we tested the compound in mice expressing TTR V30M, a model of familial amyloidotic polyneuropathy, which recapitulates the main pathological features of the human disease. Immunohistochemical and Western blot analyses showed a significant decrease in TTR burden in the gastrointestinal tract and the peripheral nervous system in mice treated with CLR01, with a concomitant reduction in aggregate-induced endoplasmic reticulum stress response, protein oxidation, and apoptosis.

Taken together, our preclinical data suggest that CLR01 is a promising lead compound for development of innovative, disease-modifying therapy for TTR amyloidosis.

Molecular cues of neuronal remodelling following spinal cord injury in a mouse model

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Objective: A common problem in patients with spinal cord injury is the loss of voluntary bladder control. Imediately after injury there is a period of spinal shock where the bladder is arreflexic and where there is urine retention. Over time an automatic voiding reflex tends to develop where the bladder muscle (detrussor) involuntarily contracts causing the individual to involuntarily leak urine throughout the day. This condition is refered to as Neurogenic Detrussor Overactivity (NDO) and the incontinence it causes has a negative impact in the lives of the patients who experience it. The goal of our project is to clarify the molecular triggers underlying the neuronal plasticity responsible for the emergence of this voiding reflex. As Trpv1, a nonselective cation channel, is known to be involved in the establishment of NDO we have analysed both wild type and Trpv1 knockout mice for changes in transcriptional activation.

Methods: We lesioned WT and KO mice by transecting the spinal cord at the lower thoracic level (T9-10) level. Cystometries were performed under deep anaesthesia induced by subcutaneous urethane. Changes in bladder preassure occur due to bladder reaching full- or near-full capacity and in response to bladder contraction which temporarily reduce bladder volume thus increasing internal preassure. This measurements can be analysed and compared for Peak bladder pressure, Amplitude and Frequency of voiding contractions. Tissue section were collected from these animals from the Dorsal Root Ganglia (DRG) and Dorsal Spinal Cord (DSC) at the S4-5 region and Spinal Cord (SC) at the lesion site (T9-T10) in Sham operated (control) and at 1 and 4 weeks post-lesion animals. The changes in expression of several transcription factors involved in development of the peripheral nervous system such as Prrxl1, Lmx1b, Tlx3, Isl1, Pax 3 where evaluated using RT-PCT.

Results: We have analysed the cystometries of WT and TRPV1 KO mice and have uncovered no differences in the emergence of NDO in the KO mice relative to WTs with TRPV1$^{-/-}$ animals having similar stages of bladder activity following spinal injury. We could find no significant differences either between both genotypes or with treatment in any of the genes tested in either DRG or DSC. We could find a significant effect of Treatment (but not of genotype) in the Prrxl1 gene in the spinal cord, which is the site of injury. In a previous report there is an implication of Prrxl1 in the context of inflamatory but not neuropathic pain in DRG neurons. Prrxl1 is a transcription factor important in the development of the peripheral nociceptive system. It seems to be involved in maintenance and possible diferentiation of glutamatergic cell populations in DRG and DSC from E14 onwards in mice.

Future Plans: In view of these results we are currently reassessing our initial assumption that the reorganization of bladder C-fiber inervation in response to spinal injury is done by re-activating developmentally-controled neuronal differentiation programs but instead focus our efforts into identifying targets of sympatic reorganization as these are the most likely responsible for the observed plasticity. We will do a candidate selection and RT-PCR analysis of molecules involved in neuronal sprouting and cytoskeletal reorganization. Our results have also opened up the possibility of Prxxl1 involvement in spinal cord injury. Our future plans include doing a more thorough histological analysis of Prrxl1 expression in sections of injured and sham-operated spinal cords to confirm Prrxl1 changes.

Hydrophobic quaternized chitosan for efficient nanoparticle formation and transfection of therapeutic oligonucleotides

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Introduction: Antisense gene therapy with the use of single-stranded oligonucleotides (ONs) holds great therapeutic potential. Especially, splice-correcting oligonucleotides (SCOs) have been recognized has capable of tackling many genetic diseases arising from mutations that produce aberrant alternative splice patterns[1]. Although the use of "naked" ONs for cell delivery *in vivo* is possible, development of delivery vectors for this specific class of molecules could further improve their efficiency, attaining a controlled delivery and decreasing toxicity levels. To this end, chitosan, a biodegradable biomaterial based vector, associated with negligible toxicity effects, holds great potential has a nucleic acid carrier, although its efficiency needs further improvements. Furthermore, its potential for delivery of ONs has not been duly investigated[2]. In this work we explored the use of a hydrophobic quaternized chitosan for ON complexation and condensation, as well as mediation of delivery of a SCO *in vitro* in a luciferase splice-correction model cell line, seen as a more reliable method for accessing delivery efficiencies of ONs by different vector types[3].

Experimental Methods: A trimethylchitosan (TMC_{43}, Kitozyme) (Mn 43.3 kDa, 11% DA, 30% quaternization (DQ)) was reacted with succinimidyl-modified stearate (NHS-SA) attaining a modification degree of 2±0.8% (mol%). TMC/ON complexes were prepared at different N/P ratios by a coacervation method aided by SO_4^{2-} crosslinking. Strength of interaction between TMC and ON was determined by gel electrophoresis assay. Size and zeta potential were determined by DLS. DLS was also used to verify the stability of formed complexes in serum containing medium. The extent of cellular association of TMC/ON complexes was analysed by flow cytometry. Transfection efficiency was evaluated by adding complexes to HeLa/Luc705 cells and analysing splice-correction of the mutated Luciferase gene in terms of increased luciferase activity[3]. All values represent means of at least 3 experiments ± SD.

Results and Discussion: TMC-SA showed slightly higher interaction strength with ONs in comparison with TMC_{43} when analysed by gel retention assay. Nevertheless, both TMCs were able to retain the ON with >90% efficiency for N/P >2. The average hydrodynamic diameter of both TMC_{43}/ON and TMC-SA/ON ranged from 100-150 nm with particles at the highest range of size given by higher N/P ratios (N/P>120). Stability of TMC/ON particles after incubation in serum containing cell medium showed a striking difference between both TMCs. While TMC_{43}/ON particles showed an increase in average size and decreased peak intensity, TMC-SA/ON particles retained almost completely the DLS profile when comparing to non-treated control samples (Fig. 1).

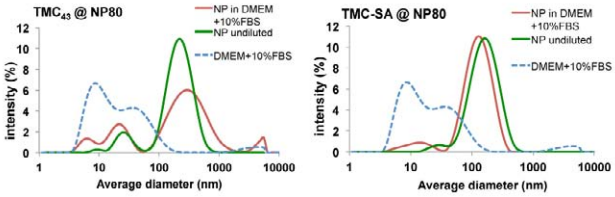

FIGURE 1: Stability of complexes in serum containing medium analysed by DLS. TMC/ON complexes at N/P 80 were incubated at 37ºC for 1h with serum containing cell culture medium.

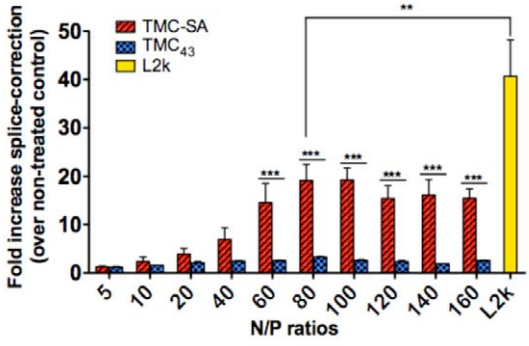

FIGURE 2: Splice correction activity of TMCs/SCO complexes in HeLa/Luc705 cell line. SCO was used at 0.3 μM final concentration. (n=3, **P=0.019; ***P<0.001)

Transfections with TMC/SCO complexes showed close to 10-fold increase in efficiency of TMC-SA in relation to TMC_{43}, and around half the efficacy when comparing to Lipofectamine™ 2000 (Fig. 2).

Flow cytometry showed similar extent of cellular interaction for TMC-SA/ON complexes and Lipofectamine pointing to other mechanisms playing a role in the transfection efficacy differences seen between both vectors.

CONCLUSION: Hydrophobic modification of TMC was found crucial for preparation of stable TMC/ON nanoparticles and produced splice-correction efficient complexes when high N/P ratios (≥80) are used. This is the first report showing the successful use of a chitosan-based biomaterial for improved delivery of a SCO in a splice correction model.

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Comparing the potency and the diffusion capacity of OnabotulinumtoxinA and AbobotulinumtoxinA after a single injection on the detrusor muscle

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Background: In the urology field the injection of botulinumtoxin type A (BoNT/A) in the urinary bladder has been approved for the treatment of several lower urinary tract disorders, such as detrusor overactivity. BoNT/A cleaves the synaptosomal-associated protein of 25 kDa (SNAP-25) and prevents the fusion of synaptic vesicles with the cell membrane and the release of neurotransmitters into the synaptic cleft. The BoNT/A serotype is commercially available in three main formulations: onabotulinumtoxinA (onabotA), abobotulinumtoxinA (abobotA) and incobotulinumtoxinA (incobotA), with the first two being the most used in clinical practice. Considering that there is no supported equivalence between the two toxin formulations, in this work, we aimed to compare the potency and the diffusion capacity of onabotA and abobotA after administration in the urinary bladder detrusor muscle.

Materials and methods: Female C57Bl6 mice were separated in three experimental groups: (1) OnabotA, (2) AbobotA and (3) Saline. All animals were deeply anesthetized and a laparotomy was performed to expose the bladder. A single dose of 0,5 U of botulinum toxin (onabotA or abobotA) diluted in 2 µL of saline was injected in the top of the bladder dome. Controls received an injection of 2 µL of saline. Three days after surgery animals were euthanized and bladders were collected and fixed. All bladders were processed for immunohistochemistry with an antibody against cleaved SNAP-25 (cSNAP-25). Immunoreactive (IR) fibers for cSNAP-25 were visualized, pictures from the entire bladder were obtained, and the number of IR fibers was quantified.

Results and discussion: Immunoreaction against cSNAP-25 was observed in the mucosa and muscular layers showing that onabotA and abobotA cleaved their target protein throughout the entire bladder. Animals injected with saline do not showed immunoreaction against cSNAP-25. Concerning the analysis of the toxin potency, we observed that the number of cSNAP-25 IR fibers in the bladder was two times higher in the bladders that received onabotA than in the ones injected with abobotA. Regarding the study of the diffusion capacity of both formulations, the number of cSNAP-25 IR fibers was counted in bladder trigones. We observed that onabotA diffusion was greater since more IR fibers were present in this bladder region, that is distant from the injection site.

Concluding message: OnabotA formulation is two times more potent and has a greater diffusion capacity than abobotA. These results show that BoNT/A formulations are not interchangeable and its clinical use should take this into account.

c-Src function is necessary and sufficient for triggering microglial cell activation

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Microglial cells are the resident macrophages of the central nervous system (CNS). Their function is essential for neuronal tissue homeostasis. After inflammatory stimuli, microglial cells become activated changing from a resting and highly ramified cell shape to an amoeboid-like morphology. These morphological changes are associated with the release of pro-inflammatory cytokines and glutamate, as well as with high phagocytic activity. The acquisition of such phenotype has been associated with activation of cytoplasmic tyrosine kinases, including those of the Src family (SFKs). In this study, using both *in vivo* and *in vitro* models, we demonstrate that among SFKs c-Src function is necessary and sufficient for triggering microglia pro-inflammatory signature, glutamate release, microglia-induced neuronal loss and phagocytosis. c-Src inhibition in retinal neuroinflammation experimental paradigms consisting of intravitreal injection of LPS or ischemia-reperfusion injury significantly reduced microglia activation changing their morphology to a more resting phenotype and prevented apoptosis. Our data demonstrate an essential role for c-Src in microglial cell activation and microglia-dependent neuronal loss.

Functional analysis of String/CDC25 phosphatase in postmitotic neurons

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String/CDC25 (Stg) is a Ser/Thr phosphatase (PPase) whose activity is associated to proliferating tissues. It is known as the universal G2/M regulator and plays a key role in cell-cycle progression by activating CDKs. The role of Stg/CDC25 in a non- proliferative context has never been addressed. However, this question is of key importance since expression of the mammalian homologues CDC25A and CDC25B has been detected in non-diseased human and mouse brains and rat primary neural cultures. The physiological relevance and the potential targets of this PPase in non-proliferative tissues are unknown. Results from our group show that Stg/CDC25 is expressed in *Drosophila* visual system neurons. The aim of this project is to uncover the function of String/CDC25 in postmitotic neurons, using the *Drosophila* as a model. Loss-of-function (LOF) studies will allow us to identify the consequences of impairment of *stg/cdc25* specifically in postmitotic neurons. In addition, a proteomics approach, two-Dimensional Differential In-Gel electrophoresis (2D-DIGE) will be used to identify direct and indirect targets of this PPase in photoreceptor neurons. This strategy will guide us to the molecular mechanisms regulated by Stg/CDC25 in a non-proliferative context. As described for other PPases (PP2A), Stg/CDC25 may also play a fundamental role in nervous system development and deregulation of its activity may be linked to neurodegenerative conditions. We will present our latest findings on the function and targets of this phosphatase.

Alternative Polyadenylation of Rho GTPases in central nervous system cells

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Alternative polyadenylation (APA) is an important regulatory mechanism occurring in 70% of eukaryotic organisms.[1,2] Proliferative cells generally select promoter-proximal polyA signals (PAS)[3] while differentiated cells and brain tissues use the distal PAS[4]. Rho GTPases are key molecular switchers essential for several cellular processes, including differentiation, and are involved in some neuronal disorders[5]. Here we show that during differentiation of rat primary oligodendrocytes, classical Rho GTPases preferentially use the proximal PAS. As classical Rho GTPases are mainly regulated by GEFs and GAPs at the protein level, it is possible that they do not require a finer regulation at the mRNA level. Interestingly, we observe the opposite APA pattern for atypical Rho GTPases, which are not regulated by GEFs or GAPs: the distal PAS is the mostly used. To address if this is a cell specific phenomenon, we tested rat primary microglia, astrocytes and neurons. In glial cell types - microglia and astrocytes - the APA pattern of both typical and atypical Rho GTPases is the same as in oligodendrocytes. In neurons however, it seems that the distal PAS is preferentially used in later stages of differentiation. A role of cell-specific APA in typical and atypical Rho GTPases function will be discussed.

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Efficacy of Novel Mesenchymal Stem Cell Populations in a Rat Model of Type 1 Diabetes-Induced Neuropathic Pain

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Diabetes mellitus is a serious chronic disorder that affects over 371 million people worldwide. Portugal, according to a recent report by the Organisation for Economic Co-operation and Development, has the highest rate of incidence (9.8%) of diabetes in the European Union (EU). Although diabetes has been the subject of very intensive research, current treatment strategies are still frequently ineffective which, over time, can lead to several complications including neuropathy.

As part of an ongoing collaborative project funded by the EU named REDDSTAR (Repair of Diabetic Damage by Stromal Cell Administration), we are currently investigating the therapeutic potential of novel bone marrow-derived mesenchymal stem cell (MSC) populations in the well-established streptozotocin (STZ)-induced Wistar rat model of painful diabetic neuropathy (DN). These novel MSC populations result from the development of a new platform technology for MSC based on the Orbsen Therapeutics Ltd. discovery of an exciting novel MSC marker, CD362. Our approach consisted in administering intravenously human heterogeneous plastic adherent (PA)-MSC, $CD362^+$ MSC, $CD362^-$ MSC, or vehicle to STZ-diabetic rats one week after STZ injection. The efficacy of the different MSC populations in preventing the development of behavioural signs of DN, namely altered nociceptive responses to mechanical and thermal stimulation, was evaluated over time. The Randall-Selitto paw-pressure test and the Hargreaves test were used to evaluate noxious mechanical nociception and thermal nociception, respectively, and were performed before STZ injection and then every two weeks until the 10^{th} week post-STZ injection. Body weights and blood glucose levels were monitored every two weeks throughout the *in vivo* experiments, and the levels of blood glycated haemoglobin A_{1C} (HbA_{1C}) were quantified at week 10 post-STZ injection.

Our results show that metabolic parameters typical of this disease model (impaired weight gain, hyperglycaemia, and elevated HbA1C levels) were not affected by intravenous injection of MCS, but behavioural signs of DN were improved. In fact, STZ-diabetic rats treated with $CD362^+$ MSC exhibited ameliorated mechanical hyperalgesia as compared to non-treated STZ-diabetic animals. Further, STZ-diabetic rats treated with any of the three MSC populations (PA-MSC, $CD362^+$ MSC, or $CD362^-$ MSC) did not present signs of thermal hypoalgesia as did their non-treated STZ-diabetic counterparts.

Our results suggest that administration of the most efficacious MSC population—$CD362^+$ MSC—may be a useful strategy to manage painful DN symptoms. It remains to evaluate the mechanisms underlying the effects of these MSC, which are likely to be mainly peripheral as the cells similarly affect two different sensory modalities.

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Adducin: the dual regulator of the neuronal cytoskeleton and of axon responses to inhibitory cues

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Although actin is well recognized as a key player in neuronal polarity and axon growth, how different actin-binding proteins control neuron biology is not fully understood. Here we studied the role of adducin, an actin-binding protein with the dual function of capping barbed actin ends and crosslinking actin-spectrin networks, including the axonal actin rings. In adducin KO mice, although axon formation is normal, neuronal migration is impaired and rupture of the external capsule occurs early in development. Axonal degeneration and enlargement arises with ageing and is prominent in the optic nerve. Further supporting a central role of adducin in coordinating the axonal cytoskeleton, *in vitro*, adducin KO neurons have increased actin and microtubule dynamics and their axon initial segment is unable to relocate following chronic depolarization. Interestingly, we observed that adducin KO neurons are capable of extending longer neurites that are less sensitive to inhibitory cues. Moreover, and further supporting the participation of adducin in the response to inhibitory signals, conditioned neurons with high growth capacity on inhibitory substrates, downregulate adducin activity both *in vitro* and *in vivo*. In sum, our data suggests a crucial role of adducin in the maintenance of the axonal cytoskeleton and in the axon response to inhibitory cues.

Acetyl-L-Carnitine prevents METH-induced disruption of Tight Junction: a role for MMP-9

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Methamphetamine (METH) is a potent psychostimulant used worldwide for its addictive properties. There is increasing evidence of METH-induced disruption of the Blood-Brain-Barrier (BBB) function and integrity. As in other neurological conditions, degradation of tight junctions (TJs) by matrix metalloproteinases (MMPs) was identified as a consistent cause for BBB disruption. Based on previous reports where acetyl-L-carnitine (ALC) administration was shown to ameliorate dystrophy and glomerular sclerosis by reducing MMP-9 activity, we hypothesized that ALC could prevent METH-induced MMPs activity contributing to maintain the integrity of the endothelial layer.

We used the bEnd.3 endothelial cell line to evaluate the neuroprotective action of ALC over METH-induced damage. Suitable METH doses were selected through viability/cytotoxicity assays. ALC 1mM was added to the cultures 30 minutes prior to METH exposure. Twenty-four hours after exposure, cells were evaluated for Tight Junctions (TJs) expression and distribution, through immunofluorescence and western blot. Zymography was used to assess MMP-9 activity and ILK knockdown was performed using shRNA-mediated silencing technology.

Our results show that METH led to disruption of the actin filaments concomitant with claudin-5 translocation to the cytoplasm. These events were mediated by MMP-9 activation in association with ILK overexpression, which may contribute to BBB dysfunction. Pretreatment with ALC prevented METH-induced activation of MMP-9, preserving claudin-5 location and the structural arrangement of the actin filaments. The present results support the potential of ALC in preserving the BBB integrity, highlighting ILK as a nouvelle target for ALC therapeutic use.

Key-words: Blood-Brain-Barrier; Methamphetamine; Acetyl-L-Carnitine; Tight Junctions; Matrix-metalloproteinase-9, Integrin-linked kinase

The oligodendrocyte "processosome": identification of new regulators of differentiation and myelination

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Oligodendrocytes (OL) are the myelinating cells of the central nervous system (CNS). During CNS development and myelination, OL extend several membrane processes that will contact and wrap axons. The formation and extension of such processes requires controlled reorganization of the cytoskeleton.

We physically separated oligodendrocyte precursor cell (OPC) soma from their processes for transcriptomic analysis (RNA sequencing) in order to identify and characterize mRNAs and microRNAs mainly present in the processes of the OPC. We hypothesize that such molecules will play important roles in OL process extension, wrapping and, later, myelination of axons.

We observed a substantial enrichment of mRNAs in OPC process related to cytoskeletal dynamics and ribosomes/translation, reinforcing the notion of local regulation of translation of mRNAs involved in oligodendrocyte process extension, as it is already described in neurons. We are currently focused on the study of three molecules, Dusp19, Kank2 and Yap1, all enriched in OPC processes when compared with soma. These participate in important signaling pathways (Akt, JNK, MAPK, Hippo, etc) that modulate the cytoskeleton dynamics and are important for OL differentiation. Nothing is known about the role of these molecules in the oligodendrocyte. We observed that the expression of these molecules is modulated during OL differentiation and knockdown experiments suggest key roles in OL differentiation. We are currently evaluating their expression during CNS development and myelination, and addressing the intracellular mechanisms and signalling pathways where they might be involved.

Finally, the microRNA sequencing revealed a substantial number of miRNAS that are enriched in OPC process. We are currently focusing on the identification of potential miRNAs that may regulate the expression of our candidates Dusp19, Kank2 and Yap1.

Altogether, this knowledge might also be relevant to better understand and promote remyelination in debilitating demyelinating diseases such as Multiple Sclerosis.

Effects of Methamphetamine in Microtubules Acetylation: preventive role for Trichostatin A

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Methamphetamine (METH) is a potent psychostimulant used worldwide for its addictive properties. A few studies have shown that METH exposure increases blood-brain barrier permeability leading to tight junctions disruption and/or cytoskeleton modifications. Microtubules acetylation is known to occur in α-tubulin, a phenomenon regulated by histone deacetylase (HDAC) 6 and strongly associated with cell dynamics and stability. HDAC inhibitors such as Trichostatin A (TSA) leads to maximum acetylation of α-tubulin and increased expression of CDKN1A. Moreover, TSA has been described as a neuroprotective agent in several neurologic diseases. The role of METH in tubulin acetylation process is still unknown.

This study aims to understand the METH impact on α-tubulin acetylation as well as on HDACs activity. Moreover, we intend to verify if TSA could be a neuroprotective agent against METH-induced effects through HDACs modulation. For this purpose, an *in vitro* model of endothelial cells was exposed to METH 1mM for 24 h, with or without the simultaneous treatment with TSA 100nM. Levels of α-tubulin acetylation were assessed through immunofluorescence and western blot and CDKN1A gene expression was evaluated as a marker of HDACs activity.

Our results showed that METH increases HDAC activity and decreases α-tubulin acetylation. Moreover, in our model, TSA was able to completely prevent METH-induced effects on microtubules acetylation.

This study reveals an effect of methamphetamine exposure that to our knowledge as never been reported. However, additional studies in a *in vivo* model are still necessary to evaluate the potential use of TSA as a therapeutic agent in METH addiction.

Key-words: methamphetamine, histone deacetylase 6, acetylation, α-tubulin, trichostatin A

Methamphetamine does not regulate the proinflammatory signature of cortical microglial cells in culture

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Methamphetamine (Meth) is a highly addictive psychostimulant drug of abuse with severe health consequences and long-term neurotoxic effects. Several studies have demonstrated that Meth induces neurotoxicity by mitochondrial dysfunction, production of reactive oxygen species (ROS), excitotoxicity and neuroinflammation.
Microglial cells, the resident macrophages of the central nervous system, play crucial roles in inflammatory processes. When activated upon inflammation, injury or disease, microglia migrate to injury sites, secrete an array of molecules including pro-inflammatory cytokines, nitric oxide and superoxide, which can cause damage to the neuronal tissue. Activated microglia are identified by their "amoeboid" morphology, acquisition a pro-inflammatory signature, release of ROS and high phagocytic activity.
Recently, microglia have been implicated as mediator of Meth-induced neurotoxicity. Besides, it has been described that Meth induces microglial cell activation in the brain of human abusers. In an attempt to clarify how Meth induces microglia activation we analyzed the effects of this psychostimulant on the phagocytic activity, production of ROS, expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory markers in primary cortical microglial cell cultures. We studied the effects of two doses of Meth (10 and 100μM) on these classical activation markers to gain further insight into microglia activity. We found that Meth significantly decreased the phagocytic activity of microglial cells while no significant changes were observed for the production of ROS and iNOS when compared with control condition. We also used quantitative RT-PCR to evaluate the Meth effects on interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF) and iNOS mRNA expression in primary microglial cell cultures 24 hours post treatment. We observed that Meth did not induce significant changes in the mRNA levels of theses pro-inflammatory markers. Overall, our results suggest that in the presence of Meth, isolated microglial cells do not acquire an activated signature.

Peripheral nervous system plasmalogens regulate Schwann cell differentiation and myelination

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Schwann cells, the myelinating glia in the peripheral nervous system (PNS) undergo several morphological and biochemical changes that are required for membrane outgrowth and wrapping of individual axons. Myelin is highly enriched in plasmalogens, a class of ether-phospholipids, which may account for 80% of the total glycerophosphoethanolamine pool. The importance of ether-phospholipids is highlighted by the severe clinical presentation of rhizomelic chondrodysplasia puntacta (RCDP), a disorder caused by impairment in the plasmalogens biosynthesis. To investigate the role of these ether-phospholipids in Schwann cell biology and myelination, we examined two mouse models with complete impairments in their biosynthesis, i.e., the Pex7 and Gnpat knockout (KO) mice. The histological characterization of sciatic nerves from KO mice revealed impaired radial sorting and myelination. Impaired myelination was also evident in *in vitro* cultures of embryonic dorsal root ganglia from Gnpat KO mice, and following sciatic nerve crush. Myelin devoid of plasmalogens was less compact and showed abnormal appositions. Myelin compaction in the absence of plasmalogens was partially accomplished through the action of myelin-basic protein (MBP), as a combined defect in plasmalogens and MBP severely affected myelination. These changes in myelin and Schwann cell morphology ultimately lead to an adult-onset severe demyelination with axonal loss. A deficiency in plasmalogens impairs AKT-mediated signaling, causing a dysregulation in GSK3β and modulates the defects observed in Schwann cell differentiation and myelination. In summary, our findings reveal the pivotal role of plasmalogens for myelination and highlight the importance of these phospholipids during neurodegeneration.

Design of biodegradable GATG dendrimers for DNA/RNA delivery

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Introduction: Gene therapy, besides treating inherited and acquired diseases, has been proposed as a powerful tool in the regenerative medicine field. Since naked nucleic acids are highly unstable and rapidly degraded by endonucleases, research efforts are being focused on the design of effective and biocompatible vectors with the ability to compact and protect them.[1]

Among the different nanocarriers, cationic dendrimers are fascinating candidates due to their globular, very branched and multifunctionalizable nanostructure, monodispersity and their capacity to complex and encapsulate nucleic acids in compact structures.[2] However, one limitation of the most currently used dendrimers for their use in regenerative medicine strategies is their non-degradability under physiological conditions that leads to cytotoxicity induced by the accumulation of non-degradable synthetic materials in the organism. Because of this, the development of biodegradable cationic dendrimers is highly desirable,[3] since they represent a way of avoiding cytotoxicity.

In this communication we present the preliminary results of the design and synthesis of a new family of biocompatible and biodegradable PEG-GATG dendritic block copolymers (PEG-bGATG) to serve as vectors of therapeutic nucleic acids.

Experimental Methods: Inspired by non-biodegradable GATG dendrons and their PEG-dendritic block copolymers (Figure 1), a new family of biodegradable PEG-bGATG dendrimers was synthesized, which repetition unit is also based on gallic acid and triethylene glycol arms, and with aliphatic ester bonds as focal points of degradation. They were characterized by NMR (^{1}H, ^{13}C, COSY and HSQC), FTIR and MALDI-TOF. Biodegradability studies by NMR were carried out at different pH, simulating physiological (PBS, pH 7.4, 37 °C) and endosomal (Acetate buffer pH 5.5, 37 °C) conditions.

Results and Discussion: One limitation of GATG dendrimers for its application in Regenerative Medicine strategies is its non-biodegradability. In order to achieve degradability, alternative synthetic routes are being explored, where the degradation is achieved through aliphatic ester bonds localized at the dendrimer branches, which are more labile than amide bonds under physiological conditions.

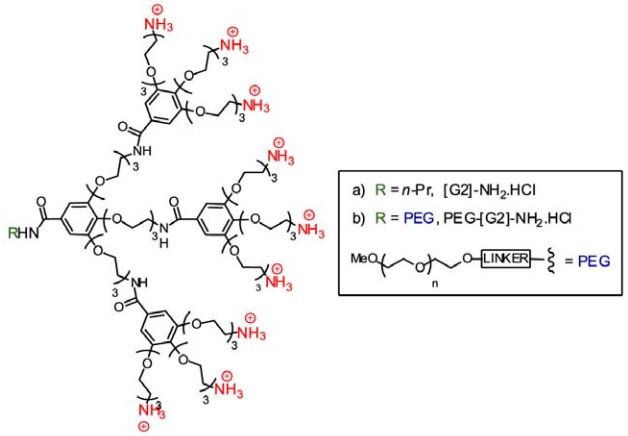

FIGURE 1: GATG and PEG-GATG dendrimers

Conclusion: Two generations of biodegradable PEG-GATG dendritic block copolymers, that will can act like vectors of nucleic acids, have been synthesized and characterized by NMR, FTIR and MALDI-TOF.

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Ceramide-activated protein phosphatase Sit4p deregulates macroautophagy, mitophagy and mitochondrial dynamics in *isc1Δ* cells

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Sphingolipids regulate crucial biological processes, such as stress response and apoptosis. The budding yeast Saccharomyces cerevisiae has been pivotal to uncover the impact of sphingolipid dynamics on eukaryotic cell physiology and metabolism. The bioactive sphingolipid ceramide can be generated by the de novo biosynthetic pathway or through the hydrolysis of complex sphingolipids, the last catalysed by the inositol phosphosphingolipid phospholipase C protein, Isc1p (the yeast orthologue of the mammalian neutral sphingomyelinase 2). Cells lacking Isc1p display shortened chronological lifespan (CLS), oxidative stress sensitivity and severe mitochondrial dysfunctions. We demonstrated that Isc1p acts upstream of Sit4p, the catalytic subunit of mammalian type 2A protein phosphatase (PP2A). The deletion of SIT4 restores mitochondrial function in *isc1Δ* cells, increasing oxidative stress resistance and extending CLS.

The regulatory role of macroautophagy in mitochondrial function has been demonstrated in *S. cerevisiae*. Since *isc1Δ* cells exhibit mitochondrial dysfunctions, we intended to assess any defect on macroautophagy impacting on mitochondrial function. The results show that the process is impaired in *isc1Δ* cells, ultimately leading to mitochondrial dysfunction. Importantly, *SIT4* deletion re-established the autophagic flux in the mutant strain, correlating with restored mitochondrial function in *isc1Δsit4Δ* cells.

Mitophagy and mitochondrial dynamics are key quality control mechanisms by maintaining a healthy mitochondrial network sustaining overall cellular energetics. We demonstrate that mitophagy was impaired during ageing and this was correlated with mitochondrial fragmentation and decreased cell viability in *isc1Δ* cells. Sit4p had also a regulatory role on mitophagy and contributed to mitochondrial fragmentation in the mutant strain.

Overall, our work demonstrates that Isc1p-driven ceramide signaling is crucial for proper regulation of macroautophagy, mitophagy and mitochondrial dynamics by Sit4p-dependent mechanisms.

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Dissecting the role of TTR proteolytic activity in Alzheimer's disease

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Transthyretin (TTR) is the plasma homotetrameric carrier of thyroxine (T4) and retinol, in the latter case through binding to retinol binding protein (RBP). TTR is mainly synthesized in the liver and choroid plexus of the brain, which constitute the sources of TTR in the plasma and in the cerebrospinal fluid (CSF), respectively. Besides its transport activities, TTR is a metalloprotease having as a substrate Aβ, the main constituent of amyloid plaques in the brains of Alzheimer's disease (AD) patients. TTR proteolysis of Aβ was proposed as a protective mechanism in AD but so far this hypothesis lacks support from in vivo data. In this work we further addressed the relevance of TTR proteolysis in AD and demonstrated that TTR proteolytic activity contributes to Aβ clearance. We measured the effect of TTR (either wt or proteolytically inactive (TTRprot-)) in the Aβ secretion using Neuro-2A neuroblastoma cells stably expressing human APP carrying the Swedish mutation (N2A-APPswe cells). We observed that while wt TTR led to a reduction in the Aβ levels secreted by N2A-APPswe cells, TTRprot- had no effect. Moreover transmission electron microscopy (TEM) analysis demonstrated that wt TTR, but not TTR prot-, is capable of interfering with A-Beta fibrillization by both inhibiting and disrupting fibril formation. To determine whether TTR proteolysis will be relevant for preventing the development of AD in vivo, we will analyse the effect of intracranial injection of recombinant TTR (either wt TTR or TTR prot-) in a mouse model for AD.

Anxiety and depression induced by monoarthritis are reversed by topical analgesia: implication of the PGI-LC-PFC pathway

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Background and aims: Pain-related anxiety and depression are common in arthritic patients. However, it is unclear if the correct treatment of arthritic symptomatology is sufficient to restore all the pain components (sensorial and emotional) associated to this condition. We aim to evaluate if peripheral analgesia is capable of reversing the centrally-mediated affective responses produced by the pain model of monoarthritis (MA) and if the Paragigantocellularis Nucleus (PGi)-Locus Coeruleus (LC)-Prefrontal Cortex (PFC) pathway is implicated.

Methods: MA induced by complete Freund´s adjuvant (CFA) was studied at 4, 14 and 28 days post-inoculation. Allodynia and hyperalgesia were evaluated by the ankle bend and paw pressure tests. Anxiety- and depression-like behaviors were assessed by the elevated zero maze and the forced swimming tests, respectively. Extracellular signal-regulated kinases 1/2 phosphorylation levels ($pERK_{1/2}$) in the PGi, LC and PFC were determined by western blot. Similar procedures were performed after induction of analgesia by topical application of Diclofenac cream.

Results: MA produced an early and stable pain hypersensitivity and inflammation. By contrast, prolonged MA (28 days) resulted in depressive and anxiogenic-like behaviors, suggesting an inability to cope with stressful situations. These behavioral changes coincided with increased $pERK_{1/2}$ levels in the PGi-LC-PFC pathway. Pain threshold, inflammation and the anxio-depressive symptoms induced by MA were reversed by Diclofenac, as well as $pERK_{1/2}$ levels in the PGi-LC-PFC pathway.

Conclusion: Prolonged MA induces affective disorders associated with $pERK_{1/2}$ expression within the PGi-LC-PFC pathway. Successful analgesia reverses sensorial and affective changes in MA rats.

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Novel biomarkers with clinical importance to increase cardiovascular risk in chronic kidney – Role of dietary sodium and phosphate intake

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Cardiovascular (CV) diseases are the leading cause of morbidity and mortality in patients with chronic kidney disease (CKD) and it is increasingly apparent that these patients are more likely to die from cardiovascular disease than to develop kidney failure. Endothelial dysfunction (ED) is an early event in CKD that precedes CV complications and may be improved by dietary restriction of both phosphate and sodium intake.

The main purpose of this study is examine how serum phosphate and volume overload affect endothelial function in CKD patients.

A representative number of CKD patients (N=100) followed-up in the outpatient clinic of Nephrology department of "Centro Hospitalar de São João" will be distributed according to the estimated GFR calculated by CKD-EPI formula. Demographic, clinical and analytical data will be recorded. Studies will be carried out in basal conditions as well as during modulation of both sodium and phosphate diet intake. The methodology will include the collection of 24h food history for quantification of the amount of phosphate and sodium ingestion in basal conditions. Patients will subsequently receive a structured eating plan including the energy value calculated according to their needs and a maximum sodium intake of 1500mg (protocol 1), 17 mg/kg/day of phosphate (protocol 2) or both regimens (protocol 3). Twenty-four hours urine samples will be collected in protocols 1,2 and 3 on days 1 and day 7 for calculation of 24h excretion of sodium and phosphate as well as for the assessment of other analytical parameters. The study aims to assess endothelial function by pulse tonometry as well as other biomarkers of ED such as circulating levels of ADMA, Renalase, FGF-23, circulating endothelial progenitor cells and volume overload (assessed by multifrequency bioimpedance) in patients with CKD included in stages 1 to 5, before and after dietary sodium and phosphate restriction.

It is anticipated that this project may provide support for the hypothesis that dietary sodium restriction and dietary phosphate restriction may both contribute to reduce the risk of CV events in CKD through the modulation of endothelial function. This may prove to be important in guiding dietary intervention strategies.

FAAH inhibition reverses bladder hyperactivity and pain induced by cystitis through a CB1 dependent mechanism.

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Introduction & Objectives: The cannabinoid system has attracted considerable interest following the demonstration that cannabis has therapeutic effects on urgency incontinence in multiple sclerosis patients. Up-regulation of endogenous cannabinoids like anandamide (AEA) through the inhibition of fatty acid amide hydrolase (FAAH), the key enzyme in AEA degradation, may offer the opportunity to activate the cannabinoid system without undesirable psychotropic effects. Here we study the effect of FAAH inhibition to treat bladder hyperactivity and pain induced by chronic inflammation. We also investigate the CB receptor subtype involved.

Material & Methods: Female Wistar rats were used. Cystitis was induced by intravesical instillation of lipopolysaccharide (LPS), 5mg/ml and experiments carried on 24h after LPS instillation. Drugs used included FAAH antagonist URB937 (URB), CB1 antagonist MJ15 and CB2 antagonist SR144528 (SR).

Cystometry were performed under urethane anaesthesia in control and inflamed animals. After stabilization drugs were given intravenously: First, URB in doses of 0.01, 0.1, 1, 5 and 10uM (cumulative doses) were tested. URB 1 uM was the most effective dose to reduce bladder reflex activity and was selected to investigate the effect of URB on bladder nociception. Pain behaviour tests were performed before inflammation (controls) , 24h after LPS instillation (LPS) and then after 1uM URB injection (LPS+URB). Another control, LPS and LPS+1uM URB rats where submitted to cystometry for 2h. Then, animals were perfused and L6 spinal cord immunoreacted against Fos. To investigate which CB receptor is involved, the effect of 1uM URB was tested in the presence of 10uM MJ15 or 0.3 mg/kg SR. Cystitis was always confirmed histologically.

Results: Frequency of bladder reflex contractions (BRC) in controls was 0.5±0.1 contractions/min and it was not changed by any URB dose. BRC of LPS rats at baseline were 2.1±0.6. URB at 0.01 and 0.1 uM decreased BRC to 1.7±0.9 and 1.2±0.5 contractions/min. URB at 1 uM completely reversed LPS-induced bladder hyperactivity (0.7±0.2 contractions/min).

Pain behaviour in LPS rats disappeared after 1uM URB. This dose decreased spinal Fos expression in L6 spinal segment of inflamed rats from 31+/-12 cells/section to 15+/-8 cells/section. This value was statistically similar to control (5+/-3 cells/section, $p>0.05$).

CB1 and CB2 antagonist, alone or with 1uM URB, did not change BRC of naïve animals. In inflamed rats, URB conserved the effect in the presence of CB2 antagonist SR144528. However, URB lost the effect in the presence of CB1 antagonist MJ15.

Conclusions: FAAH inhibitor reverses inflammatory bladder hyperactivity and pain through a CB1 receptor mechanism. These results may be highly relevant for the clinical use FAAH antagonist.

Intrathecal administration of botulinum toxin type A improves urinary bladder function and reduces pain in rats with cystitis

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Background: Botulinum toxin A (Onabot/A) has been shown to have an antinociceptive effect. This might be due to an impairment of sensory nerves not only in the peripheral but also in the central nervous system. In this work, we analysed both systems by studying the effect of intrathecal (i.t.) administration of botulinum toxin A in an animal model of bladder pain and hyperactivity induced by cyclophosphamide (CYP).

Methods: Rats were implanted with an i.t. catheter at the L6 segment. Bladder pain was induced by intraperitoneal (i.p.) injection of CYP. Five experimental groups were created: (1) Saline i.p. + i.t.; (2) Onabot/A i.t.; (3) CYP i.p. + saline i.t.; (4) CYP i.p. + Onabot/A i.t. 48 h after CYP; and (5) Onabot/A i.t. 30 days. Mechanical sensitivity was assessed in the abdomen and hindpaws. Motor activity was observed in an open-field arena. Bladder reflex activity was evaluated by cystometry. At the end, bladders and spinal cord were
immunoreacted (IR) against cleaved SNAP-25 (cSNAP-25), c-Fos, p-ERK, calcitonin gene-related peptide (CGRP) and GAP43.

Results: The toxin reduced pain symptoms, bladder hyperactivity, expression of neuronal activation markers and CGRP, typically up-regulated in this inflammatory model. The presence of cSNAP-25 was detected in the spinal cord and bladder fibres from animals treated with Onabot/A. No somatic or visceral motor impairments were observed.

Conclusions: Our findings suggest that i.t. Onabot/A has a strong analgesic effect in a model of severe bladder pain. This route of administration can be further explored to treat intractable forms of pain.

Amyotrophic lateral sclerosis, palliative care and mental health

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Amyotrophic lateral sclerosis (ALS) is a rare progressive, fatal motor neuron disease (MND) throughout the central nervous system. The mean age of onset varies between 58–63 years for sporadic disease and 47-52 years for familial disease. Median survival time is about 2-3 years. Its pathophysiology has emerging evidence of a complex interaction between genetic and molecular pathways. The main presentations of ALS include limb-onset ALS with a combination of upper and lower motor neuron signs in the limbs and bulbar onset ALS, presenting primarily with speech and swallowing difficulties. Patients experience progressive denervation and atrophy of skeletal muscles and in most cases die from respiratory failure. Moreover, there is already clinical, neuropsychological, genetic, imaging, and neuropathological evidence that non-motor systems are affected in ALS. Recently, the ALS research community adopted the concept that ALS is a multi-system disorder associated not only with MND, but also with frontal and temporal lobar dysfunction. In the context of this severe multi-system disorder, there has been a significant amount of research concerning its psychiatric manifestations, mainly mood and anxiety disorders reactive to adaptation process. Also, there has been some evidence about psychiatric manifestations assumed to be consequent to neurodegeneration, mainly personality change and behavioral symptoms. Therefore, authors propose to make a psychopathology, personality and behavioral assessment of a group of patients with ALS at baseline and at follow-up (1 year), through psychiatric interview and psychological scales approved for portuguese population. Also, authors intend to make neuroimaging characterization of patients and establishment of possible correlations between collected data.

Psychiatric manifestations in ALS may have a serious impact on interpersonal relationships, care taking, but also in treatment adherence and in decision making, which makes them important areas of research. Characterization of them, besides providing valuable insights, can offer clues to improve care and clinical assistance to these patients, highlighting the role of psychiatrists in promoting better quality of life for patients and significant ones.

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The role of T cell receptor N-glycosylation in Inflammatory Bowel Disease pathogenesis and progression

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Inflammatory Bowel Disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract that includes Ulcerative Colitis (UC) and Crohn´s disease (CD). IBD remains an incurable disorder with an increased risk to evolve to colorectal cancer[1,2].

The incidence of IBD is increasing worldwide and it is therefore of paramount importance to identify and characterize the underlying molecular mechanisms of IBD pathogenesis in order to improve the development of novel biomarkers that may help the determination of prognosis and also improve the patients´ stratification for appropriate treatment. Several reports support that T cell activity and signaling is tightly regulated by GnT-V-mediated glycosylation[3,4].

In this study we aimed to address whether the (dys) regulation of the interplay between N-glycan branching and T cell activity is a major contributory factor and a yet uncovered mechanism underlying UC. Our results showed that UC patients exhibit a clear dysregulation of the GnT-V-mediated glycosylation of the T Cell Receptor (TCR) on intestinal lamina propria T cells, which has been associated with T cell hyperactivity and hyperimmune response. Patients with severe UC showed the most pronounced defect on N-glycan branching in mucosal T cells. We further showed that UC patients (with active disease) exhibit a deficiency in T lymphocytes MGAT5 gene transcription comparing with normal controls, which underlies the observed dysregulation of T cell glycosylation. This deficiency in T cells N-glycosylation was demonstrated to accompany disease severity and progression[5].

Overall, the disclosure of this new molecular mechanism in UC disease opens new windows of opportunity to further explore the potential applicability of this mechanism in predicting disease course and risk to evolve to colorectal cancer.

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An *in vivo* model for Intervertebral Disc Regeneration

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Intervertebral disc (IVD) degeneration is responsible for various spine pathologies and present clinical treatments are insufficient. Concurrently, the exact mechanisms behind IVD degeneration are still not completely understood, so as to allow development of efficient tissue engineering approaches. In this study it is proposed a minimally invasive model of rat IVD degeneration and herniation, by disc needle puncture injury. Two different needles gauges were used: a low caliber 25-G needle and a high caliber 21-G needle. Histological, biochemical and radiographic degeneration was evaluated for both groups at 2 and 6 weeks post-injury. We show that the larger caliber needle results in a more extended histological and radiographic degeneration within the IVD, respect to the smaller caliber needle. TUNEL quantification also indicates higher amount of cell death in the 21-G group. Analyses of collagen type I (Picrosirius red staining) and type II (immunofluorescence) and GAG content (Blyscan assay) all seem to indicate that degeneration features spontaneously recover from 2 to 6 weeks, for both needle types. Moreover, we show hernia formation for both needle gauges, where the hernia dimension is proportional to the needle gauge and the number of macrophages present in the site, identified by CD68 immunofluorescence, is proportional to the hernia size. These results may help understand the complex mechanisms behind IVD pathophysiology.

Post-Transplant Bone Disease: A Prospective Bone Biopsy Study

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Background: Post-transplant bone disease results from a combination of factors, which include previous bone and mineral metabolism disturbances, and effects from transplant-related medications. Bone biopsy (BB) remains the gold-standard diagnostic tool.

Methods: 6 patients (5M/1F, age 52.5±9.7, on RRT for 56.5±10.2 months) were prospectively studied after renal transplantation (RT). Dual-X-ray absorptiometry (DXA) and trans-iliac BB after double tetracycline labeling were performed at baseline (BL) in the first 2 months after RT and after 2 yrs of follow-up (1 pt after 5 yrs). We used Paired t-tests and Pearson correlation in statistical analysis.

Results: Comparing to BL biopsy, 2nd biopsy revealed a significant decrease in Ob.S/BS (0.65±0.47 to 0.06±0.13%, p=0.035), ES/BS (3.72±2.21 to 1.87±1.10%, p=0.025), Oc.S/BS (0.63±0.34 to 0.40±0.62%, p=0.035), N.Ob/BS (0.46±0.29 to 0.07±0.18/mm2, p=0.043). Although a decrease in BV/TV did not reach significance, a significant decrease in TbN (2.29±0.42 to 1.68±0.46/mm, p=0.008) and increase in TbSp (390.53±95.84 to 530.87±163.37µm, p=0.029) in the 2nd biopsy suggest loss in bone quantity. We also did not find significant differences between OV/BV, OS/BS and Oth. All BB demonstrated a marked reduction in BFR, and so no difference was seen between the 1st and 2nd. At T0 3/6 patients had PTHi>300pg. The scarce tetra labeling in the 1st BB was probably a result of high dose corticosteroids in the first weeks after RT, which can lead to miss-interpreted results. Mlt was 4 times higher in the 2ndBB, although not significant, possibly due to small number of patients. At BL, DXA lombar parameters strongly correlated with OV/BV (DMO r=0.822, p<0.05; T score r=0.853, p<0.05; Z score r=0.942, p<0.01). At follow-up BB, this correlation is lost, possibly due to effects of transplant medications on bone density. We found no correlation with femoral site.

Conclusions: The results observed in this population show a reduction in bone activity, namely at cellular level, suggesting increased risk of adynamic bone and loss of bone volume.

Mechanisms involved in endothelial damage and dysfunction in Lupus Nephritis

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Background: Cardiovascular disease (CVD) is a well-recognized complication of Systemic Lupus Erythematosus (SLE). Although overall mortality for patients with SLE has improved owing to advances in treatment and better understanding of disease mechanisms, mortality due to CVD has remained high.

Traditional risk factors do not fully explain the increased risk of cardiovascular disease in SLE patients. Therefore, disease-related as well as non-traditional risk factors have been postulated in this population.

Lupus nephritis (LN), one of the most severe manifestations in SLE, occurs in up to 60% of adults. The development of LN predicts the risk of chronic kidney disease (CKD) and poor survival and is an important factor for accelerated atherosclerosis as well.

Aims: The main objective of this study is to evaluate in LN patients the influence of disease-and CKD-related factors in premature atherosclerosis.

A cross-sectional study design will be carried out. For such purpose, it will be assessed endothelial function in three different populations of patients (SLE with LN, SLE without LN and CKD patients without any rheumatic systemic disease or corticosteroid use) with pulse tonometry and biomarkers such as circulating levels of ADMA, FGF-23 and circulating endothelial progenitor cells for evaluation of endothelial function. Vascular calcifications will be also evaluated by quantitative score using multislice computed tomography and by a semi-quantitative evaluation by simple vascular calcification score based on plain radiographic films of pelvis and hands.

As secondary objectives, this research aims to characterize CKD-MBD in LN patients by bone histomorphometry and clarify its influence in endothelial dysfunction (ED) and vascular calcification as well.

Expected outcomes, translational relevance and impact: This research aims to improve the CVD risks stratification of LN patients by focusing on biological and imaging surrogate markers of subclinical atherosclerosis in attempt to identify atherosclerosis at the earliest stage and to elucidate for future therapeutic approaches.

Caracterização da osteodistrofia renal em doentes renais crónicos prevalentes em diálise peritoneal e em doentes renais crónicos pré-diálise tratados com diferentes captadores de fósforo

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Os distúrbios do metabolismo ósseo e mineral na doença renal crónica (DRC) são uma importante causa de morbilidade e de calcificações extraesqueléticas que têm sido associadas a um aumento na mortalidade cardiovascular. O termo osteodistrofia renal deve ser reservado para as alterações na morfologia óssea encontradas em doentes renais crónicos e é quantificável por histomorfometria óssea.

O espectro da osteodistrofia renal na diálise peritoneal (DP) permanece por definir. Os estudos que avaliaram este tema são escassos, têm vários anos e reportaram os seus resultados através de nomenclatura desatualizada. Uma parte do estudo consiste em caracterizar as diferentes formas de osteodistrofia renal em doentes prevalentes em DP da Unidade de DP do Centro Hospitalar de São João (CHSJ). Para tal, propomos-nos a realizar um estudo de coorte transversal, com o intuito de avaliar a osteodistrofia renal em doentes tratados exclusivamente por DP há pelo menos 6 meses, através de realização de uma biópsia óssea transilíaca com avaliação histomorfométrica.

A hiperfosfatemia está associada a um aumento na mortalidade entre os doentes dialisados e também na DRC pré-diálise. Existem diversos tipos de captadores de fósforo, entre os quais, hidróxido de alumínio, sevelamer e carbonato de cálcio. Não existem estudos que tenham avaliado a utilização do hidróxido de alumínio como captador de fósforo na pré-diálise, nomeadamente através de biópsias ósseas com avaliação histomorfométrica. A restante parte do estudo consiste em avaliar a segurança e eficácia do hidróxido de alumínio como captador de fósforo na DRC estadios 3 e 4. Será realizado um estudo de coorte prospectivo, observacional, com o intuito de comparar o hidróxido de alumínio com sevelâmero e carbonato de cálcio.

Espera-se que este trabalho permita ao grupo de investigadores retirar ilações acerca das alterações ósseas encontradas nos doentes em DP bem como da eficácia e segurança do hidróxido de alumínio no tratamento da hiperfosfatemia na pré-diálise.

Serum phosphate and FGF-23 blood levels independently predict peritoneal daily phosphate removal in patients on peritoneal dialysis

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Introduction and aims: Fibroblast growth factor 23 (FGF23) levels increase early in the course of chronic kidney disease and help maintain phosphate serum levels despite reduced renal function. The aim of this study was to test the hypothesis in patients on peritoneal dialysis that FGF23 serum levels may be interrelated with peritoneal daily phosphate removal independently of phosphate serum levels and other confounders.

Methods: Sixty-eight adult patients (male 43%, mean age 47±13 years, mean time on PD 8.7 months, range 1 to 72 months, 95.5% on continuous ambulatory PD [CAPD], 4.5% on automatic PD [APD]) were evaluated in a cross-sectional study (ST1). A subgroup of 33 patients (100% on CAPD), mean time on PD 8.2 months, was reassessed 12 months after the first evaluation (ST2). Daily phosphate intake was assessed by food frequency questionnaire. Peritoneal daily Pi removal (PDPiR) was calculated from 24-hour peritoneal effluent. Intact FGF-23 blood levels were assessed by ELISA (Immutopics, Inc., San Clemente, CA).

Results: In ST1, FGF-23 serum levels positively correlated with serum Pi (r=0.526, p<0.001), dietary phosphate intake (r=0.286, p=0.0.44), serum urea (r=0.327, p=0.010), serum creatinine (r=0.509, p<0.001), PDPiR (r=0.607, p<0.001), and negatively correlated with age (r=-0.362, p=0.003) and residual renal function, RRF (r=-0.333, p=0.007). PDPiR positively correlated with serum phosphate (r=0.734, p<0.001), iPTH (r=0.391, p=0.001), peritoneal creatinine clearance (r=0.340, p=0.005), FGF-23 (r=0.607, p<0.001) and negatively with RRF (r=-0.487, p<0.001). In a multiple linear regression analysis, both FGF-23 and Pi serum levels closely predicted PDPiR (β=0.302, p=0.012 and β=0.587, p<0.001 respectively) independently from other confounders.

In ST2, a significant decrease in RRF was observed during the 12 months follow-up period (p<0.001) going along with a significant decrease daily urine phosphate removal. This was accompanied by an increase in FGF23 without changes in Pi serum levels. FGF-23 serum levels positively correlated with PDPiR on both the 1st (r=0.509, p=0.003) and the 2nd (r=0.578, p=0.001) evaluation periods.

Conclusions: FGF-23 blood levels and serum phosphate are both closely associated with peritoneal daily phosphate removal in patients on PD. This is observed independently other confounders such as RRF, dietary phosphate intake, iPTH. It is suggested that serum Pi and FGF23 may regulate peritoneal phosphate transport through different mechanisms.

Volume overload predicts Vascular calcification in peritoneal dialysis patients

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Introduction and aims: Vascular calcification and arterial stiffness are non-traditional risk factors of cardiovascular disease in patients on peritoneal dialysis (PD). On the other hand, disorders of volume status are increasingly recognized as contributors to hypertension and adverse cardiovascular outcomes in this population. We hypothesized that fluid overload may be related with vascular calcification in PD patients.

Methods: Sixty-five adult patients (male 37, mean age 47 years, mean time on PD 8.8 months, range 1 to 72 months, 95.5% on continuous ambulatory PD [CAPD], 4.5% on automatic PD [APD]) were evaluated in a cross-sectional study. Body composition and phase angle was measured by tetrapolar BIA (InbodyS10, Biospace, Korea) at 50 kHz. Arterial stiffness was evaluated by augmentation index measured by digital pulse amplitude tonometry (Endo PAT, Itamar Medical). Vascular calcification was assessed by simplified calcification score, described by Adragão *et al.* (*Nephrol Dial Transplant* 2004,19)

Results: In the linear correlation analysis PhA was positively associated with serum albumin (r=0.434, p<0.001), serum urea (r=0,410, p= 0,001) and serum creatinine (r=0.358, p=0.005); and negatively associated with age (r=-0.352, p=0.007), augmentation index (r=-0.289, p=0.04), calcification score (r=-0.473, p<0.001) and extracellular water/total body water ratio [ECW/TBW] (r=-0.857,p<0.001). In a multiple linear regression analysis, PhA was a independent predictor of augmentation index (β=-0-388,p=0.03) independent of serum Pi levels, serum calcium levels, iPTH, age, systolic and diastolic blood pressure. Among patients with calcification score >3, lower PhA (EXP(B)=0.369,p=0.01) and higher ECW/TBW (EXP(B)=2.422,p=0.034) were associated with higher vascular calcification score independent of serum Pi levels, serum calcium levels, iPTH, age, systolic and diastolic blood pressure

Conclusions: Volume overload predicts vascular calcification in patients on PD. This relationship occurs independent of serum Pi and calcium levels, iPTH, age as well as both systolic and diastolic blood pressure. Our results highlight the importance of achieving optimal volume status in PD patients.

Phase angle – a technique to assess nutritional status

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Introduction and aims: Protein-energy malnutrition is a risk factor for mortality in dialysis patients. Phase Angle (PhA) evaluated by multi-frequency bioelectrical impedance analysis technique (BIA) has been increasingly used not only for the assessment of the hydration status but also to evaluate changes on cellular membrane integrity, two aspects that may highlight nutritional assessment. However, the role of PhA for the assessment of malnutrition in peritoneal dialysis (PD) patients still remains to be established.

Methods: Sixty-one adult patients (male 37, mean age 47 years, mean time on PD 8.8 months, range 1 to 72 months, 95.5% on continuous ambulatory PD [CAPD], 4.5% on automatic PD [APD]) were evaluated in a cross-sectional study. Phase angle, body composition and hydration status were measured by tetrapolar BIA (InbodyS10, Biospace, Korea) at 50 kHz. The nutritional status was evaluated by the subjective global assessment [SGA] and biochemical parameters.

Results: The mean PhA of the studied population was 5,8° and 28,3% of patients had PhA < 5°. In the linear correlation analysis PhA was positively associated with serum albumin (r=0.434, p<0.001), urea (r=0.410, p=0.001), creatinine (r=0.358, p=0.005), intercellular water (r=0.443, p<0.001) and fat free mass (r=0.379, p=0,003) and was negatively associated with age (r=-0.352, p=0.007) and extracellular water/total body water ratio [ECW/TBW] (r=-0,857 p<0.001). In a linear multiple regression analysis, low PhA predicts higher ECW/TBW (β=-0,008, p<0,001) independently from residual renal function, 24h urine volume, ultrafiltration volume, systolic blood pressure, serum albumin and age. When the nutritional status was assessed by SGA, mean PhA was significantly higher in patients classified well-nourished (n=27) in comparison with those classified malnourished (n=34) (6.5°±0,9 vs 5,2°±1.4, p<0.001). In addition, well nourished group had lower ECW/TBW than malnourished group (0,377±0,001 vs 0,386±0,1 p<0,005)

Conclusions: We found a close relationship between higher PhA measured by BIA and better nutritional status evaluated by body composition, biochemical parameters and SGA. In PD patients low PhA is a direct predictor of higher ECW/TBW. The indices obtained from BIA, especially PhA, showed to be a robust and fast method to the assessment of nutritional and hydration status of PD patients.

“Browning” of White Adipocytes: Melanocortins as Essential Players

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Introduction: White adipose tissue (WAT) stores lipids whereas brown adipose tissue (BAT) burns fat to produce heat. The discovery of beige or brown-like cells within WAT set off the conversion of white into brown adipocytes as an attractive option in the management of obesity. Beige adipocytes arise by transdifferentiation of white adipocytes when subjected to certain conditions, such as chronic β-adrenergic stimulation, exposure to cold and PPARγ ligands. The aim of this study was to investigate if the melanocortin neuropeptides also have the ability to induce similar changes in white adipocytes, promoting their differentiation in beige / brown adipocytes.

Methods: After stimulation of 3T3-L1 adipocytes with the melanocortin alpha-MSH the expression of UCP-1, Resistin, and Cidea genes were measured through real time PCR. The Perilipins phospho-activation was determined by western blotting and its cellular localization by immunofluorescence assay. The rate of lipolysis was assessed by quantification of the levels of non-esterified fatty acids (NEFAs) and glycerol in the culture medium. The mitochondrial quantification was estimated by binding of the fluorochrome 10-N-nonyl acridine orange.

Results: Adipocyte stimulation with alpha-MSH increased the expression of UCP-1, characteristic of beige / brown adipocytes and decreased the expression of resistin, a white adipocyte marker. Mitochondria biogenesis was significantly stimulated after 24h. Interestingly, Cidea expression decreased, despite its association with BAT. However, in WAT, Cidea delimits lipid droplets such as Perilipins, protecting from lipolytic enzymes. Decreased expression of Cidea is therefore consistent with the increased levels of lipolysis promoted by alpha-MSH, Perilipins phosphorylation and its dissociation from lipid droplets.

Conclusions: The melanocortin alpha-MSH appears to induce beige / brown adipocyte characteristics in 3T3-L1 adipocytes.

S100a9 calgranulin is consistently over-expressed in CD8+ T-lymphocytes from Hfe deficient mice and patients with Hereditary Hemochromatosis

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Lymphocytes were described as modifiers of iron overload in several immunodeficient mouse models and in the clinical context of Hereditary Hemochromatosis (HH). However, the molecular players involved in the iron-lymphocyte interactions are still unknown. Here we aimed to identify candidate genes differentially expressed in CD8+ T-lymphocytes from HH relatively to controls.

Total RNA from CD8+ T-lymphocytes from the HH murine model Hfe-/- and from C57Bl/6J controls was extracted and whole genome comparative gene expression using Affymetrix Exon ExprChip.Mouse Gene 1.0ST was performed. Genes with the highest differences in expression between the two mouse models were selected as candidate genes. The results were validated by qRT-PCR in mice and humans.

Affymetrix results showed for the first time a clear expression of the S100a8 and S100a9 calgranulins genes in CD8+ T-lymphocytes. These were also the most differently expressed genes, respectively 17 and 13× up-regulated in Hfe-/- versus controls. The same result was observed whether the animals were maintained with diets with normal or high iron contents. In order to address the physiological relevance of the previous results, expression of calgranulins were tested in CD8+ T-lymphocytes, from HH patients and controls, by qRT-PCR and flow cytometry. At the mRNA level the expression of S100a9 in culture conditions were systematically higher in HH patients than in controls ($p=0.007$). At the protein level this results was confirmed with HH patients showing an expression of S100a9 significantly higher in comparison with controls ($p=0.003$).

In conclusion, we show for the first time that S100a8 and S100a9 are expressed in human CD8+ T-lymphocytes and that their expression, namely of S100a9, is upregulated in the context of HH. The biological significance of these findings is currently being addressed, namely the relative roles of iron overload or the lack of functional HFE in the modulation of these novel candidates.

Carotid atherosclerosis evaluation in a cohort irradiated in childhood for tinea capitis treatment

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In the 5th and 6th decades of the past century children infected with tinea capitis were submitted to X-ray scalp epilation. The dose to the neck region, namely the carotid arteries, was a low dose, estimated to be around 100mGy. Having had access to a Portuguese cohort of individuals irradiated in childhood with this therapeutic approach, we decided to evaluate carotid atherosclerotic disease as a possible long term side effect of the X-ray scalp epilation.

So far we have clinical examined 283 irradiated individuals and 174 non-irradiated individuals, mainly spouses of the irradiated ones. The irradiated individuals are being randomly selected from a cohort of 1375 previously clinically examined for head and neck neoplasias (thyroid and basocelular carcinomas). All the individuals perform the B-mode ultrasound imaging of carotid arteries for intima media thickness and stenosis evaluation according to a predefined protocol, and a panel of blood serum measurements, including a lipid profile, homocysteine, HbA1c, hsCRP, and hepatic enzymes. In the clinical examination the clinical record is registered, blood pressure is measured, and anthropomorphic measurements are obtained, such as waist circumference (WC) and body mass index (BMI).

Our preliminary results show that the irradiated individuals had more frequently plaques in the left carotid comparing to the non-irradiated ones (40.4 *vs* 28.2, p=0.009). For the right carotid the difference was not significant (34.7 *vs* 29.4, p=0.25). Moreover, in the irradiated group, the individuals that developed plaques had significant higher white blood cells and hepatic enzymes, which were not observed in the non-irradiated group. Contrarily, in the non-irradiated group, the significant differences between the individuals with plaques and without plaques where mainly in the traditional cardiovascular risk factors, namely hypertension, diabetes, waist circumference, and smoking load, not observed in the irradiated group.

Although the results obtained are still preliminary, being this an ongoing project, it appears that the low dose irradiation context may induce a different pattern of carotid atherosclerosis development.

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Knowing disease: patients first

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The health field has been witnessing a redefinition of the disciplines that structure it, which results in a rearrangement of the knowledge and skills of their agents. This evidence is expressed in variable degrees and points to a more diligent and informed attitude of patients in the management of their illnesses. However, this tendency does not display enough strength in order to interfere with the internal dynamics of health/disease domain. This paper reports on a multidisciplinary project entitled "Knowing disease: patients first", developed in the Institute of Molecular Pathology and Immunology at the University of Porto (IPATIMUP, Portugal) and funded by Calouste Gulbenkian Foundation. Usually, the biomedical perspective strictly aims to reset the state of health but it does not incorporate the multiple dimensions that the illness experience encloses throughout its extent. This study explores the multidimensionality in this issue through the discourse analysis mostly of oncological patients, using a strategy of action inquiry framed in a contemporary approach of Grounded Theory methodology. The population is composed of 110 individuals of both genders who accessed consultations by specialists of two public hospitals in Porto. Semi-structured interviews have been conducted with 100 patients with breast, lung, colon, esophagus and prostate cancers, as well as 10 patients with benign lesions of breast and prostate. The interviews were performed in two moments. The script of the first is based on McGill Illness Narrative Interview (MINI), designed to stimulate illness narratives in health research. The second interview, whose script was originally developed by the team of the project, aims to collect participants' evaluation from a set of Frequently Asked Questions concerning pathology reports. Qualitative analysis has been performed with NVivo 10 software. Socio-biographical profile of the population was also collected in order to contextualize the information obtained from patient narratives in their social conditions, symbolic and material resources and respective paths of life. Grounded theory methodology assisted us to develop a model concerning the personal and social construction of oncologic disease, and produced findings with potential usefulness to patients, professionals and researchers.

Alpha$_{1A}$-adrenoceptor antagonist suppresses bladder pain and urothelial disruption in a model of bladder pain syndrome/ interstitial cystitis (BPS/IC) induced by chronic adrenergic stimulation

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Introduction: Bladder painful syndrome/ interstitial cystitis (BPS/IC) is a chronic debilitating condition, characterized by suprapubic pain, urinary frequency and urgency, mostly predominant in women. The etiology of BPS/IC remains unknown. It has been shown that BPS/IC patients present an increased sympathetic activity, characterized by an increase in tyrosine hydroxylase expression in the bladder, increased noradrenaline levels in urine and elevated mean blood pressure and heart rate during bladder hydrodistention. Sympathetic overactivity can be observed in several human painful diseases and in rat pain models. The observed increase in sympathetic activity and high levels of urinary noradrenaline presume an overstimulation of adrenergic receptors present in the urinary bladder. The urinary bladder expresses different subtypes of α-adrenoceptors and β-adrenoceptors. It has been suggested that TRPV1 is overexpressed in the bladder of patients with BPS/IC. Furthermore, it was also shown that TRPV1 receptor is essential for pain arisal and increase in bladder frequency during cystitis.

Previous studies showed that chronic adrenergic stimulation in rats induced pain behavior, increased voiding frequency and urothelial disruption that mimic BPS/IC. Pain and increased urinary frequency where dependent of capsaicin sensitive primary afferents activation since desensitization of these fibers with capsaicin reverse the phenylephrine mediated effect, demonstrating a possible relation between adrenergic activity and nociceptive activity. Hence, the aims of this study was demonstrate the involvement of adrenoceptors in the appearance of BPS/IC symptoms, determine which alpha1-adrenoceptor (AR) subtype were implicated in the process, understand if phenylephrine acts at central or peripheral level and cross-talk between adrenoceptors and nociceptive receptors.

Methods: A group of adult female Wistar rats were injected with 2.5mg/Kg/day phenylephrine (PHE; s.c. for 14 days) alone or concomitantly with 0.2 mg/Kg/day silodosin (predominant α_{1A}-AR antagonist), with 0.9 mg/Kg/day naftopidil (predominant α1D-AR antagonist) or with 0.05 mg/Kg/day prazosin (α1-AR antagonist) (n=6 for each group). Visceral pain behavior and alterations in organ motility were analyzed before and 14 days after PHE treatment. A group of animals were submitted to a surgical intervention to put a intrathecal catheter link to one minipump with PHE for 14 days. Visceral pain behavior and cystometry were performed in these animals. At day 15, animals were anaesthetized with urethane (1.2 g/Kg) and cystometries were performed for 2h. Animals were then perfused with paraformaldehyde and the bladder and L6 spinal segment were harvested. To analyze urothelium morphology, bladder sections were stained with hematoxylin-eosin and immunoreacted (IR) against caspase 3 (to investigate urothelial apoptosis). Bladder sections were stained with Toluidine Blue to study mast cell infiltration. L6 spinal cord sections were immunoreacted against Fos protein. To study the cross-talk between adrenoceptors and nociceptive pathways, a group of TRPV1 knockout mice (n=4) were injected with PHE daily for 14 days. A control group with wild type mice injected with saline solution and a group of wild type mice injected with PHE were used. Visceral pain behavior was analyzed. To verify if PHE enhances TRPV1 response, adult female Wistar rats (n=4) was injected with PHE for 14 days. At the end of treatment, animals were submitted to a cystometry. After saline infusion, 50 μM capsaicin was infused. This procedure was repeated in another group of four animals injected with saline, as a control group.

Results: PHE induced visceral pain behavior which was counteracted by silodosin and partially counteracted by naftopidil and prazosin. PHE induced and increase of bladder reflex activity from 0.4 ± 0.1 to 1.9 ± 0.9 bladder contractions/ minute, which was reversed by silodosin (0.7 ± 0.2 bladder contractions/ minute), naftopidil (0.5 ± 0.1

bladder contractions / minute) and prazosin (0.7 ± 0.2 bladder contractions/ minute). PHE induced urothelial disruption in 88.3 ± 7.9% of urothelium length. Silodosin decreased this length to 33.9 ± 15.3%. Naftopidil and prazosin did not ameliorate the length of urothelial disruption (79.1 ± 14.3% and 46.9 ± 17,7%, respectively). The areas of urothelial disruption showed an intense caspase 3-IR. PHE induced mast cell infiltration (17 ± 6 cell/ mm^2) in the suburothelial layers. Silodosin and prazosin reduced the mast cell infiltration (14 ± 4 and 16 ± 8 mast cells/ mm^2). Naftopidil did not ameliorate mast cell infiltration (20 ± 5 mast cells/ mm^2). At L6 spinal cord level, silodosin, naftopidil and prazosin reverse the PHE-induced increase in the number of spinal Fos expressing neurons from 33 ± 5 to 17 ± 5, 12 ± 5 and 3.7 ± 0.5. Animals that received intrathecal PHE, did not presented pain behavior nor bladder activity alterations.

In TRPV1 knockout mice, injection of PHE did not induce pain behavior.

Administration of capsaicin to control animals increase bladder contractions from 0.4 ± 0.1 to 1.1 ± 0.1 bladder contractions/ minute. Administration of capsaicin to PHE treated animals increase the number of bladder contractions from 1.73 ± 1.02 to 2.65 ± 1.32 bladder contractions/ minute.

Conclusions: Chronic adrenergic stimulation with PHE induces visceral pain, mast cell infiltration, apoptosis of urothelial cells and activation of nociceptive pathways. Silodosin seem to be more effective than naftopidil to reduce bladder pain and urothelial dysfunction induced by chronic adrenergic stimulation. These results may be relevant for the clinical use of specific $alpha_{1A}$-adrenoceptors antagonists in BPS/IC. Preliminary results of intrathecal administration of PHE showed that PHE don´t act at central level. This work also shown that TRPV1 are involved in bladder hyperactivity.

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Evaluation of circulating endothelial progenitors cells by multicolor flow cytometry a prognostic and predictive microvasculature regeneration biomarker in patients with chronic kidney disease

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Background: Endothelial progenitor cells (EPC) are small immature precursor cells, detectable in plasma and bone marrow, with a physiologic role in the vascular and tissue regeneration. To date, EPC numbers have been correlated with the numbers of cardiovascular risk factors, extent of coronary disease, and future cardiovascular events. The absolute number, or functional capacity of EPCs, was suggested to be reduced in several disease states including chronic kidney disease (CKD). EPCs may be a potential mechanism by which vascular integrity is compromised, increasing cardiovascular disease risk and contributing to renal disease progression in CKD.

Circulating EPCs play important roles in accelerating endothelialization at areas of vascular damage, and EPC enumeration is a viable strategy for assessing reparative capacity. However, there is no standardized methodology of EPCs analysis or consensus on their phenotype. Moreover, published data on this subject is often conflicting and obtained from heterogeneous studies.

Based on the previous considerations, we hypothesized that the assessment of EPCs levels could represent an important prognostic and predictive biomarker of endothelial dysfunction in CKD population.

Aim: Because there are no specific markers for EPCs, the main objective of this study is the establishment of a standardized protocol to EPCs identification in peripheral blood using multicolor flow cytometry technique.

Methods: A 20-ml sample of venous blood will be used for the isolation of endothelial cells (ECs) and endothelial progenitor cells (EPCs), identified by flow cytometry (BD FACSCanto™ II system, BD Biosciences) using a combination of fluorochrome-conjugated primary antibodies for their specific cell surface markers: CD31, CD34, CD45, CD133, CD117, CD146, and CD309. Due to the rare nature of EC and EPCs, a large number of events will be collected, at least 500,000 to 1 million. Exclusion of dead cells will be done according to a fixable viability dye staining. CD34 is a cell surface markers express in hematopoietic stem cells and both ECs and EPCs. Identification of ECs will be done by double $CD31^+/CD146^+$ labeling. Identification of EPCs will be performed by $CD45^-/CD117^+/CD133^+/CD309^+$ multiple labeling.

Expected outcomes, translational relevance and impact: Taking into consideration that endothelial dysfunction can be reversed or improved by lifestyle modifications and medical therapies, it is anticipated that the assessment of circulating EPCs in CKD population could help to select the patients most likely to benefit from guiding intervention strategies that may have the potential to improve cardiovascular health by inducing vascular protection.

Ethics review of animal experiments across the European Union under the Directive 2010/63/EU

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The European legislation regarding the protection of animals used for scientific purposes, in place since 1986 (Directive 86/609/EU), has been recently revised, updated and expanded.

The new European Directive (Directive 2010/63/EU, from 22 September 2010, with full effect in MS on 1 January 2013) covers new aspects such as the review and authorization procedures in the field of project evaluation. According to the Directive, the project evaluation should consist in the assessment of the projects' i) goals and predicted scientific benefits or educational value, ii) compliance with the 3R's (Reduction, Refinement and Replacement), iii) severity, iv) harm-benefit analysis and v) need for retrospective assessment. These aspects correspond to what is conventionally referred as "ethical review", although this specific term is not explicitly used in the Directive. Despite the detailed information about what should be covered in the project evaluation, where and by whom projects should be evaluated was left to individual MS to determine.

Within the ANIMPACT Project (http://animpact.net/), a European research project that intends to provide an ethical and practical perspective of the impact of the Directive 2010/63/EU, we are mapping the ethical review process in the European Union (EU). At present, we have completed the mapping of EU-15 and we are gathering information about the new MS. This mapping is based on information available on the internet and provided by key contact persons.

We have found wide diversity in the *geographical organization* of the ethical review process, which may take place on a national, regional/local or an institutional level. In most MS, *committees* are clearly the predominant approach and it seems that systems in which individual experts perform the evaluation are on the way out. The *composition* of the committees shows both similarities and differences between MS: while scientific and animal welfare/veterinary expertise is present in all MS and legal or ethics experts are present in several MS, genuine lay representation is very rare and in most cases committee members from outside the research community are representatives of special interest groups, in particular animal protection NGOs.

In the poster presentation we will present the main results of this mapping and discuss the potential underlying reasons for the different approaches as well as its consequences for the ethical review process.

Acute effect of a phenylalanine-free amino acid mixture on metabolic parameters – from basic science to clinical Phenylketonuria

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Phenylalanine (Phe)–free amino acid mixtures (AAM) have been unequivocally effective for the Phenylketonuria (PKU) patients. Notwithstanding, metabolic effects of free amino acids contained in AAM are still not well studied, particularly metabolic responses following AAM versus intact protein. Taking into account that free amino acids are well-known insulin secretagogues, insulin has a pivotal role in glucose homeostasis and glucagon-like peptide-1 (GLP-1) presents insulinogenic effects, it seems relevant perceive to what extent AAM ingestion can trigger such responses.

Objectives: To compare *in vivo* (animal model) metabolic responses to Phe-free amino acids (present in the AAM) versus intact protein, administered acutely.

Design and Methods: Wistar rats received by gavage a bolus of Phe-free amino acids from AAM (n=7) and intact protein as albumin (n=7), diluted in a glucose solution. Blood drops were used to measure glucose at time 0 (baseline) and 15, 30, 60 and 120 min after gavage and blood was collected at the same time points to determine serum insulin. Pancreas and distal ileum/proximal colon were collected for subsequent analysis by immunohistochemistry and Western blotting, respectively.

Results: The main finding of this study is that AAM improves glucose tolerance. Glucose and insulin peaks occurred at 15 min and higher 120 min insulin levels were displayed in controls compared with treated rats, accompanied by significantly higher pancreatic insulin content as well as higher intestinal proglucagon and GLP-1 expression.

Conclusion: This study suggests that amino acids from AAM play a crucial role in glucose homeostasis and this effect is possible mediated by GLP-1.

KEYWORDS: Phenylketonuria; Amino acids; Glucose; Insulin; Glucagon-like peptide-1

Células Progenitoras Endoteliais no Transplante Renal

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Introdução: A disfunção endotelial tem vindo a ser reconhecida como marcador precoce de ateroesclerose subjacente ao aumento da mortalidade CV que se observa na DRC. Neste contexto, existem vários estudos recentes que atribuem relevância no papel das células progenitoras endoteliais (CPE) como marcadores de risco CV, de função e da reparação endotelial. Nos doentes submetidos a transplante renal, a contribuição das CPE para a melhoria da função vascular é mal conhecida e com resultados controversos.

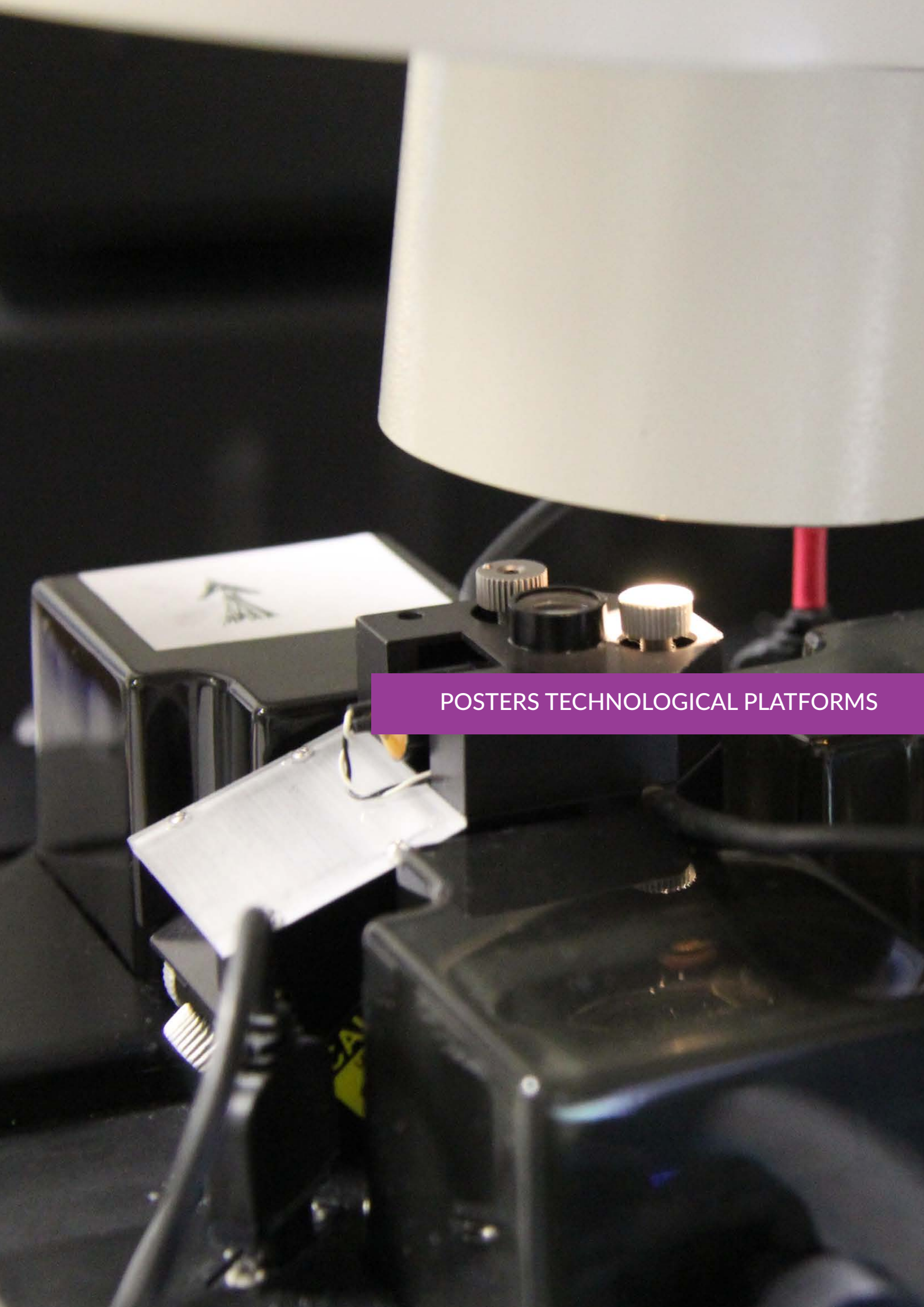
Objectivos: Avaliar prospectivamente os níveis de CPE no transplante renal de dador vivo (TRDV) tentando esclarecer o significado das CPE na função endotelial, nas populações de dadores e receptores e sua relação com o grau de disfunção renal, terapêutica imunossupressora, análogos da vitamina D, tempo de isquemia, rejeição e outros fatores de co-morbilidade.

Métodos: Avaliação prospectiva dos níveis de CPE no TRDV, antes e após a cirurgia, tendo como controlos, dois grupos:

Grupo 1: dadores vivos, sem co-morbilidades, em que os níveis de CPE serão avaliados prospectivamente, antes e após a uninefrectomia, independentemente do tempo de isquemia e na ausência de terapêutica imunossupressora. O estudo deste grupo de dadores permitirá, para além de funcionar como grupo de controlo dos receptores, esclarecer a possibilidade de que a doação de rim possa estar a associada, por si própria, a aumento de morbilidade e mortalidade CV nos dadores, a médio/longo prazo;

Grupo 2: doentes com DRC submetidos a transplante de cadáver, em que os níveis de CPE serão avaliados prospectivamente, antes e após o transplante e em que a influência do tempo de isquemia será avaliada independentemente da terapêutica imunossupressora e de outras co-morbilidades. Em todos os grupos os níveis de CPE serão correlacionados com outros marcadores de função endotelial e de risco CV na DRC, designadamente a função endotelial avaliada por tonometria digital, os níveis de FGF-23, os níveis de Células Endoteliais Circulantes e os níveis de ADMA.

Discussão: O número e função das CPE são hoje considerados importantes marcadores de risco CV nos doentes renais crónicos em pré-diálise, hemodiálise e no pós-transplante renal. O transplante renal é a melhor alternativa terapêutica de substituição de função renal e o de dador vivo é aquele com melhores resultados de função e sobrevida de enxerto renal. Para além de permitir analisar, nestas populações de dadores de rim em vida, os respectivos receptores e ainda comparando esta última população com receptores de cadáver, a contribuição das CPE como marcadore de risco cardiovascular acelerado, através da análise da sua associação com a morbi-mortalidade e com outros factores de risco CV, a aplicabilidade dos resultados potenciais deste projecto está relacionada com o facto de estes marcadores poderem virem a ser úteis na compreensão dos mecanismos de regeneração, de isquemia e nas situações de lesão renal aguda.



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TECHNOLOGICAL PLATFORMS | POSTER 01

Students' perception of e-learning in Laboratory Animal Science training

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With an increasing need of training in Laboratory Animal Science, e-learning appears to be a promising solution to issues of limited time and resources. As e-learning is increasingly used, it is important to understand how students perceive this approach to learning. At IBMC we have integrated e-learning in our advanced (FELASA Category C) training, as a complement to classroom lectures (blended learning) and in our introductory (FELASA Cat B) course (theoretical part exclusively delivered by e-learning). We assessed participants' acceptance of the e-learning platform and level of satisfaction of its use during the last 3 years with E-learning Acceptance (QELA), a concordance Likert-type scale. This study included 127 participants (60% from the C course), 21 to 50 years old (M=28.42; SD=6.22), of which 76% were women, mainly PhD students and postdocs.
Results revealed that participants from advanced and basic courses show, in general, a positive acceptance to this approach (94.4%), and a very positive perception of the platform usability (70.9%). In both courses participants strongly agreed that e-learning was useful for time management (71.4%). Moreover the majority of the participants recognized that e-learning had a positive influence in practical classes with animals (66.1%).

TECHNOLOGICAL PLATFORMS | POSTER 02

Advanced Light Microscopy – Screening Center @IBMC (ALM-SC)

André F. Maia & Paula Sampaio
Instituto de Biologia Molecular e Celular (IBMC)

The ALM-SC aims to make available to the scientific community a total solution for high content analysis (HCA) and high content screening (HCS) in basic and applied research. Providing the technology, laboratory facilities, analysis software, assay development and validation required by project teams to successfully run medium-to-high throughput screens.

TECHNOLOGICAL PLATFORMS | POSTER 03

The role of the advanced flow cytometry unit in the life science research

C. Leitão[1], A. Carmo[1]
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The Advanced Flow Cytometry Unit (AFCU) has the mission to offer efficient and reliable cytometry services based on the researchers' needs. Scientific and technical consultancy; training; data analysis; sorting and quality control are among the services provided by the AFCU. The facility has 2 flow cytometers (Calibur and Canto II) and 1 sorter (FACS Aria I).
The permanent dialogue with the researchers allows to identify their needs and to pursue for solutions. Some examples are:
- Purchasing optical filters that permit the distinction of both GFP and YFP in the same sample.
- Upgrading the Aria I with the laser 561nm that makes possible the analysis and sorting of cells expressing red fluorescent proteins.
- Improving researchers' protocols to increase resolution and get good data analysis
- Finding alternative setting controls to spare cells from the sample
Ultimately, flow cytometry is a technology widely used in many different applications in life science (including the 3 scientific areas of I3S) that will be presented in this poster.

TECHNOLOGICAL PLATFORMS | POSTER 04

Development of a fast diagnosis kit for leishmaniasis

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Leishmaniasis is a disease caused by a protozoa, genus *Leishmania* spp. and affects humans and dogs. There are three clinical-pathological patterns for that disease: cutaneous (CL), mucocutaneous (MCL) and visceral (VL), caused by at least 20 species. This disease is endemic in 88 countries such as India, Brazil, Sudan, Pakistan, Afghanistan, Bangladesh, Nepal, and Ethiopia, with an annual incidence of about 1.5-2 million people, being responsible for a yearly death toll of 50000. Organism prevalence differs by geographical distribution. In the Mediterranean basin and South America, leishmaniasis is a zoonosis which the main disease reservoir are the *Leishmania infantum* infected dogs. In endemic areas, the seroprevalence among dogs can reach 40%. These numbers are considered underestimated due to the fact that the reference serological techniques currently used have limitations in detecting asymptomatic humans and animals. According to the World Health Organization, the control and elimination of the disease (canine leishmaniasis) in the natural reservoir will contribute to the eradication of human leishmaniasis. The rapid diagnosis of the disease on the field is extremely important to control its spread. However, the endemic areas of this disease are located in third world countries or in the developing countries. It is therefore crucial to develop a portable and inexpensive diagnostic kit that can be operated by unskilled individuals. This constitutes an advantage for an easy and user-friendly in third world or in the developing countries. Thus, the main goal of this project is to develop an innovative portable screening method for symptomatic and asymptomatic serodiagnosis of leishmaniasis in dogs and humans. This method will be based on disposable sensors (i.e. probe) for immunorecognition assay based on green nanomaterials.

TECHNOLOGICAL PLATFORMS | POSTER 05

Quality Management to improve research

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The management of the Quality System at INEB and its continuous improvement has been a constant throughout the time and it is achieved by the use of several quality tools. These tools allow the adequate management of the implemented procedures and guarantee that the whole quality system serves its purpose in the laboratory.
This continuous improvement is achieved by:
-Ensuring the development, implementation and management of the quality documentation (registries, equipment's technical procedures, general procedures);
-Detection, management and evaluation of unconformities, in order to establish the appropriate corrective and/ or preventive actions;
-Ensuring the periodic quality control of the assays by the use of certified standards and equipment monitoring registries;
-Establishment of the metrological control plan, through the periodic calibration and/ or verification of the equipment and ensuring its accomplishment;
-Evaluation, selection and approval of suppliers, concerning the qualification in the supplied services;
-Evaluation of the SUIM clients' satisfaction in order to meet the clients' needs and promote the improvement of the services;
-Update of the Equipment File and management of the Lab Books utilization;
-Evaluation of the lab users' suggestions;
-Update on new legislation and standards related to the Quality System.

TECHNOLOGICAL PLATFORMS | POSTER 06

Protein production and Biomolecular analysis@ Porto: from knowledge to support

Frederico Silva
IBMC

The Protein Production and Purification Unit (UP3) of the Institute for Molecular and Cell Biology (IBMC) in Porto provides access to know how and facilities for the expression, purification and biochemical/biophysical analysis of recombinant proteins. The facility provides both scientific and technical expertise, aiming at the establishment and support of interdisciplinary research and networking activities involving internal and external research groups.
Users have access to facilities for the heterologous expression of proteins in prokaryotic and eukaryotic systems, as well as to chromatographic techniques, and to a wide range of analytical methods such as spectrofluorimetry, circular dichroism spectrometry, microcalorimetry and surface plasmon resonance. Proteins, as well as a wide variety of other biomolecules (such as nucleic acids and small molecules), can be studied in terms of their specific activity, stability, and molecular interactions.
The facility centers its efforts in the build-up, maintenance and spreading of know-how in its areas of expertise. In this presentation the work of the platform, from technologies implementation to support of research and organization of courses, will be portrayed.

TECHNOLOGICAL PLATFORMS | POSTER 07

Ipatimup Proteomics Unit: Research Activities and Services for the Scientific Community

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Ipatimup Proteomics Unit was created in April 2007 with the aim to provide investigators, namely from I3S, access to proteomic approaches. More information is available at www.ipatimup.pt/proteomics

The Unit is equipped with an MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, AB SCIEX) optimized for protein identification and protein post-translational characterization. This Unit also has the necessary equipment for protein separation by 1D and 2D gel electrophoresis and protein expression analysis/quantitation.

It is integrated at RNEM, the Portuguese Mass Spectrometry Network. RNEM was recently recommended for inclusion in the FCT's Research Infrastructures Roadmap in category 1 (ref. ROTEIRO/0028/2013).

Our Unit is prepared to perform the following studies:

1. Mass analysis of intact proteins, peptides, metabolites or polymers.
2. Protein identification by the Peptide Mass Fingerprint technique, PMF (MALDI-TOF) complemented by peptide sequencing/fragmentation (PMF+MS/MS, MALDI-TOF/TOF).
3. Peptide de novo sequencing by MALDI-MS/MS.
4. Protein post-translational modifications (PTMs) characterization, namely phosphorylation and glycosylation.

The IPATIMUP Proteomics Unit has scientifically participated in studies related with protein identification, and protein post-translational modifications characterization together with I3S researchers [1-3].

It has also developed glycoproteomic methodologies to identify novel cancer-associated glycoprotein biomarkers in the serum of gastric cancer patients [4,5].

We have also provided proteomics services to the scientific community with emphasis to I3S researchers. 6 research groups of IPATIMUP, 15 research groups of IBMC, and 2 research groups from INEB, have sent samples for analysis on a regular basis.

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TECHNOLOGICAL PLATFORMS | POSTER 08

Radioactivity Safety

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Supervised by the Radiation Safety Committee, we provide guidance to the IBMC.INEB Associated Laboratory through our commitment to health and safety. By employing best practices and collaboration, we promote a productive and safety-conscious work environment. We assist in the safe use of ionizing radiation including radioactive materials (RAM) and x-rays, the protection of users and environment from exposure, and ensure the safe receipt, handling, use and storage of RAM, as well as safe disposal of radioactive wastes. Our goal is to facilitate safe conditions for utilizing radiation, maintain radiation exposures As Low As Reasonably Achievable (ALARA), and to ensure operations are in compliance with applicable regulations, such as medical surveillance, radiological dosimetry, and facilities and equipment licensing.

New methodologies for protein production

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Expression and purification of correctly folded proteins demands the screening of different parameters such as: DNA constructs variants, solubility enhancing tags, *Escherichia coli* strains, media composition and culture conditions (e.g. temperature). The biggest challenge is to find an optimal combination of parameters with a minimum of resources.

Hence we are developing and/or implementing higher throughout the various stages of recombinant protein production and purification: cloning, expression screening and protein purification.

In order to increase the efficiency of the initial cloning step a recent method to customize protein expression vectors for fast, efficient and background free parallel cloning was implemented.

Sequence and ligation independent cloning (SLIC) is a cloning method that allows the assembly of multiple DNA fragments simultaneously in a single reaction using *in vitro* homologous recombination and single-strand annealing. The expression vector series, pCoofy share the same pET backbone but encompass different tags in addition to the HRV 3C protease cleavage site. High cloning efficiency is guaranteed via strong constitutive *ccdB* expression that, in contrast to DpnI digestion, is 100% efficient in counter selection of parental insert-less vector.

The next step in the protein production pipeline is the scouting of the optimal expression conditions. To that aim we need to work with tens of proteins/constructs thus a high-throughput expression screening at a small scale (1–3 ml of culture in 24 deep well plates) is being implemented to identify the optimal conditions for soluble protein production. Cell lysis is carried out in a ultrasonicator (Diagenode™), to test different working buffer compositions followed by a small scale purification chromatography using micropipette tips (e.g. Phynexus™) on the recent acquired liquid handling systems of the Advanced Light Microscopy Unit.

The UP3 has also implemented eukaryotic protein expression using insect cells and Baculovirus vectors. Both individual proteins and well as multiprotein complexes, which have become an intensive focus of research in recent years, can be expressed in this system. The baculovirus-insect cell expression system dubbed Multibac, yields considerable quantities levels of protein (typically hundreds of ug to several mg per liter), significant eukaryotic post translational protein processing capabilities and localization to the correct cellular compartment.

Other protein production methods under implementation will be showcased.

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TECHNOLOGICAL PLATFORMS | POSTER 10

Zebrafish, a new vertebrate model for scientific research at I3S

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In the past decades the zebrafish has become a powerful biological model for several scientific research fields such as developmental biology, neurobiology, pharmacology, genetics and cancer research. The advantages of a zebrafish-based research cover aspects such us: low-cost maintenance of a vertebrate model, knowledge of the genome, well-organized scientific community and an excellent experimental and genetic accessibility due to the biological characteristics of these animals.

Indeed, characteristics such as the external fertilization, rapid development and optic transparency of the embryos, enables *in vivo* observations and allow a diversified range of methodologies.

The short generation time and the capacity to produce hundreds of progeny per week are valuable characteristics of the zebrafish for studies involving genetic screens. Furthermore, their homology with mammals and their regeneration capacity makes them extraordinary models to approach biomedical questions with a strong impact in human disease research. With the aim to introduce this model to the IBMC- scientific community, the Zebrafish Facility at IBMC ("O Zebrário") was launched in December 2013 under the direct supervision of the Vertebrate and Regeneration research group (PI: J. Bessa). The facility is currently outfitted with a ZebTec Zebrafish housing system, containing two recirculating racks, with the maximum capacity of 100 tanks (3.5L). Water pH and salt levels are continuously measured and automatically adjusted and high water quality is maintained by an incorporated filter system. The current production of embryos allows the maintenance of transgenic lines and the realization of innumerous molecular and genetic studies and is used by 2 research teams. In summary, the "Zebrário" represents a new dawn in the *in vivo* study of vertebrate biology at I3S, offering new capabilities such as high-throughput screenings (e.g. chemical or genetic), low cost genetic and transgenic assays and development of hight-tech tools for basic research.

TECHNOLOGICAL PLATFORMS | POSTER 11

Genomics Core Laboratory

José Luis Costa, Patrícia Oliveira, Ana Mafalda Rocha, Carla Oliveira and José Carlos Machado
IPATIMUP

The Genomics Core Laboratory is a full service facility dedicated to provide investigators state of the art technological solutions in the field of genomics and high throughput bioinformatics data analysis. We offer technical expertise and support with experimental design, protocol development, data analysis and interpretation. Training is another crucial aspect of our work; we can tutor individual researchers and organize practical courses on the covered topics.

The laboratory is equipped with capillary electrophoresis sequencers (Applied Biosystems 3130xl genetic analyzer and ABI Prism 310 Genetic Analyzer), real-time PCR systems (Applied Biosystems 7900HT Fast Real-Time PCR Systems), digital PCR system (QuantStudio 3D digital PCR system), a system to automate RNA and DNA sample QC and quantification (Agilent 2200 TapeStation system and Qubit 2.0 Fluorometer), a sonicator for both DNA and Chromatin shearing (DiaGenode Bioruptor), an automated system for next generation sequencing library preparation (Ion Chef) and two next generation sequencers (Ion PGM system and Ion Proton System).

The laboratory is proficient in providing: small genome sequencing, exome sequencing, targeted DNA and RNA sequencing, metagenome sequencing, Sanger sequencing and digital PCR for validation purposes. Protocols for whole transcriptome sequencing and bisulphite sequencing are currently under development. Bioinformatics pipelines have been established for every sequencing service, and can be applied according to the users' requests. As for experimental design, all bioinformatics' results can be discussed and interpreted to maximize scientific outputs.

Additionally, due to the very dynamic and rapidly evolving field of genomics, we actively collaborate with different companies involved in developing NGS related products both for sample preparation and for bioinformatics data analysis. The laboratory is an Ion Torrent certified service provider.

Services Unit for Interfaces and Macromolecules: from micro to nano world

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The Services Unit for Interfaces and Macromolecules (SUIM) is a facility of INEB - Instituto de Engenharia Biomédica, which activity started in 2004.

The objective of SUIM is to provide assays of high quality level both to internal researchers and to the external community (either in the research or in the industry areas). This facility includes advanced physical, chemical and structural characterization techniques, particularly dedicated to the surface study of materials, down to the micrometric and nanometric level, in the fields of interfaces and macromolecules. The SUIM services are suitable for the study of the interaction between surfaces and interfaces of materials with cells and tissues.

The quality of the services provided by SUIM is improved year by year due to the Quality System implemented at INEB. The system allows the adequate management of the procedures implemented and guarantees that the assays are performed within strict rules. This continuous improvement is achieved by: the establishment of the metrological control plan, through the periodical calibration and/ or verification of the equipment and ensuring its accomplishment; the implementation and management of documents; the detection, management and evaluation of unconformities, in order to establish the appropriate corrective and/ or preventive actions; the evaluation, selection and approval of suppliers, concerning the qualification in the supplied services; the evaluation of the SUIM clients' satisfaction in order to meet the clients' needs and to promote the improvement of the services; the up-date on new legislation and standards related to the Quality System among others. The figure 1 presents the number of assays performed in the period of 2005 to 2013.

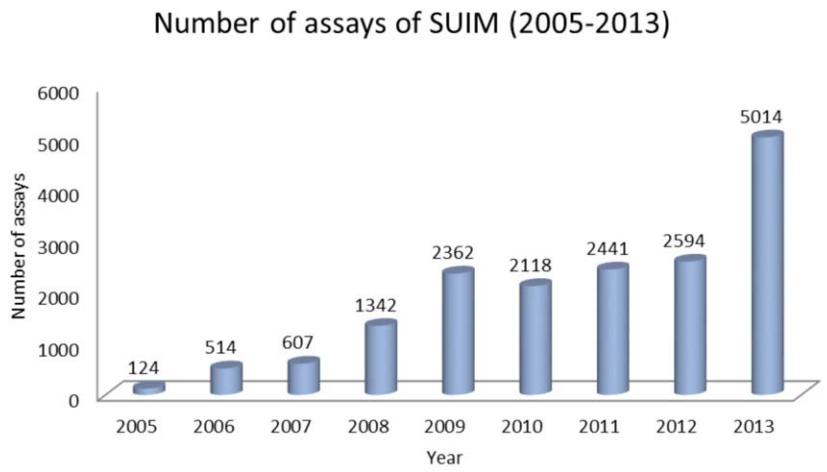

FIGURE 1: Samples analysed at SUIM (2005 – 2013)
(*) Data extracted from the SUIM report activity of 2013

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b.IMAGE - Bioimaging Center for Biomaterials and Regenerative Therapies

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The Bioimaging Centre for Biomaterials and Regenerative Therapies (b.IMAGE) is part of the Instituto de Engenharia Biomédica (INEB), a center with a wide experience and recognition in the biomaterials and biomedical imaging fields. The b.IMAGE was created in 2010 following a successful application to structural funds within the framework of the ON2 program (2007-2013 North Regional Operational Program; SAIECT-IEC/2/2010). The b.IMAGE operates on the basis of core projects and programs at the bioimaging/ biomaterials and/or regeneration interface.

As a center of competences, the aim of the b.IMAGE is to **advance in the development, improvement, integration and use of bioimaging solutions through research, technology development and education, in the fields of Biomaterials and Regenerative Medicine.** We provide high quality *in vitro* and *in vivo* imaging equipment for the biological, biomedical and bioengineering community, being involved in the development and implementation of new techniques and applications, helping also the users in experimental design, data acquisition and analysis, as well as equipment usage. The equipment available at our Centre allows for the study of complex biological questions going from preclinical studies in small animals (micro-ultrasound system) through tissues and cells (laser scanning confocal microscope and imaging flow cytometer) down to molecular complexes and molecules (Atomic Force Microscope and Raman Confocal Microscope). Examples of the applications in use are: non-invasive real-time *in vivo* micro-imaging of small animals by combination of ultrahigh frequency with a high-resolution digital imaging, for simultaneous and in real-time acquisition of anatomical, functional, physiological and molecular information down to 30 µm; 3D image acquisition of cells and tissues, colocalization, excitation fingerprinting, FRET, FRAP, photoswitching and photoactivation; high resolution imaging of high content of cells in flow with 6 different channels for every event acquired combined with a high versatility analysis software for multiple data analysis; acquisition of 3D topographic image and surface roughness, at the nanometric scale, as well as adhesion force determination by force spectroscopy; non-destructive chemical imaging of unlabeled samples for biomaterials and biological samples with a resolution down to the optical diffraction limit (~200 nm).

The Centre is also involved in post-graduate training by participating and organizing workshops and Symposiums in the fields of bioimaging, biomaterials and regenerative therapies.

In order to improve our service and to increase the different methodologies and techniques, we look forward to receive your input (b.image@ineb.up.pt).

Location: INEB- Instituto de Engenharia Biomédica, Universidade do Porto, Rua Campo Alegre, 823, IPATIMUP – Instituto de patologia e imunologia Molecular, Universidade do Porto, Rua Roberto Frias s/n.

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In vivo CAM Assays Unit (IPATIMUP) Research activities and services using the chick embryo as an animal model

Marta Teixeira Pinto
Head of the unit; Ipatimup - Institute of Molecular Pathology and Immunology of the University of Porto, Portugal

The chick embryo has long been used as a model organism in a number of research areas including oncobiology and biomaterials. The chick embryo is surrounded by the chorioallantoic membrane (CAM), a highly vascularized extra-embryonic membrane that can be used to graft human cells. When grafted on CAM, tumor cells are capable of stimulating the formation of new blood vessels, gaining their blood supply. This allows them to develop in a similar manner as in their natural hosts, i.e. to proliferate, invade and metastasize to the chick embryonic organs. The *in vivo* chick embryo model presents several important advantages (in relation to rodents) including natural immunodeficiency, easy access, short experimental duration, low cost and few animal experimentation ethical concerns.

Ipatimup's *in vivo* CAM assays unit started in 2012 with the aim of providing researchers access to this *in vivo* tool. We are prepared to perform studies to evaluate the angiogenic, tumorigenic and/or invasive response of tumor cells. These assays were designed to validate cancer target molecules (e.g. genes and proteins) in an *in vivo* context. In addition, we designed assays for drug screening based on cell xenografts. These can be treated with various candidate drugs to assess their effect on tumor induced angiogenesis, growth and/or invasion.

We have participated in several research projects [1-8] and provided services to various research groups in IPATIMUP and INEB. We also established collaborations with other national and international research centers.

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TECHNOLOGICAL PLATFORMS | POSTER 15

Cell Culture and Genotyping Service (CCGen)

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CCGen collaborated with 30 IBMC/INEB research groups, Animal House, CGPP core facility and with other research institutes, CIBIO, FMUP, FFUP, FCUP and IPO in Porto, for the improvement of cell culture conditions, genotyping and gene expression techniques.. The monitored rooms for cell culture lab were used by 17 research groups and by IPO . Genotyping and gene expression service provided the use of real-time PCR equipment for a total of 1211 PCR runs; 25 researcher groups, from IBMC/INEB and FCUP, used this equipment. With Experion (automated electrophoresis system) about 2000 RNAs were analysed for IBMC/INEB, and for CIBIO in a total of 175 chips performed. With automated extractor (Maxwell16) 127 DNA samples were extracted for 2 research groups of IBMC/INEB. The Nanodrop 1000 equipment was used by 166 researchers, from 31 research groups, in a total of 2183 utilizations for quantification of DNA, RNA and Protein. Nine new protocols of mouse genotyping service were implemented and optimized allowing the routine analysis of 74 different mouse genes and a total of 33864 genotyping. All our services offer technical assistance to all researchers. The service works with different groups of the IBMC/INEB as seen in the acknowledgments of different scientific articles published.

TECHNOLOGICAL PLATFORMS | POSTER 16

HEMS - Histology and Electron Microscopy Service

Rui Fernandes
IBMC

This Core Research Facility is centered on Transmission Electronic Microscopy (TEM) and Optical Microscopy (OM) with the ancillary equipment.
The Facility provides both the equipment and technical support to researchers needing high level optical and TEM to tackle studies either of cell or material sciences.

Besides the instruction of researchers the Facility also offers training courses as well as guided visits to high school students. Currently the Facility is engaged on workshops or exhibitions for the general public organized by the Office of Science Communication of the IBMC.

Clinical management of animal health in laboratory rodent colonies

Lamas, Sofia[1]
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Laboratory animal disease prevention and detection is an important task in an animal facility considering the ethical and scientific demand on maintaining animal quality and result consistency. Laboratory rodent diseases are associated with genetic, environmental and microbiological causes.
Genetic and environmental causes are less frequent however, microbiological causes are still an important cause of disease. The implementation of a rederivation system at the IBMC animal facility allowed us to reduce the number of cases related to microbiological agents, however these are still being the main cause of disease in other areas of our facility, still not rederived. Microbiological agents cause many times subclinical disease and their impact is especially important in immunodepressed animals or in genetically modified animals since infections can result in premature death or clinical disease. Microbiological control programs have an essential role in the prevention of infectious disease in laboratory animals. Regardless of the disease cause, detection of problems is fundamental, since in most of the cases diseases interfere with the experimental results. Early detection of disease is a key point to reduce the spread of infectious agents and to avoid clinical manifestations of disease. Detection of animals with clinical signs is first done by the caretakers and then a veterinary observation is also performed. Treatment in laboratory animals is a challenge. The size of the animal, the experimental demands or the scientific value of the animal can limit our treatment options. To improve the detection of problems and the rapid communication of the treatment plan or endpoint establishment, a software application is being used to manage on a daily basis the critical animals at the facility. A database of clinical problems is being developed which will allow us in the future to calculate precise disease incidences at the facility. Animal identification in the software is introduced by the caretaker team, followed by veterinary observation and decision making. Researchers also have access to the application and to the clinical cases related to their strains.

POSTERS ADMINISTRATIVE PLATFORMS

01
Make Data The Canary In Your “Coal Mine”
José L.R. Sousa, Pedro Martins, Andre Torres, Hilário Gil Magalhaes, Julio Rodrigues, Barbara Amorim

02
Occupational Health and Safety
J. Correia

ADMINISTRATIVE PLATFORMS | POSTER 01

Make Data The Canary In Your “Coal Mine”

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The big data era promises a third wave to pave the way to a personalized understanding of information systems entanglements, with technosocial models and measurements helping to conceptualize some of organizational contexts. Despite the extraordinary progress in understanding the technology, its use and adoption at organizational level still remains on of the information systems management problems. New approaches to this entanglement are required and possible. The IBMC Information Systems Department supported by an Emergeise framework signifies a new era in personalized information systems, which synthesizes technological advances in technology and complex-networks modelling. A truly personalized information systems imprint of the organizational micro-environment and subsequent conceptual and development insight is gained, with the ultimate goal of matching the “right” sociomaterial entanglement to the “right” business goal identifying predictive elements for information systems evolution. This comprehensive mapping of information systems entanglement structure in tandem with crucial functional annotations for organizational processes provides unprecedented insight and predictive power for information systems management. Overall, we show that major information systems development in tandem with changes in information systems thinking have laid the foundations for the Emergeise approach and the future big data application for a truly sociomaterial approach to information systems conceptualization.

ADMINISTRATIVE PLATFORMS | POSTER 02

Occupational Health and Safety

J. Correia[1]

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Occupational Health and Safety promotes a culture of safety, health and environmental protection in collaboration with the IBMC.INEB Associated Laboratory to support education and research. The main goal is the elimination of work-related illnesses and injuries by improving workplace health and safety programs, by providing information, training, education, management and solutions that support health, safety and wellness. For these matters, OHS provides direct support both in planning for and responding to emergencies, emphasizing protection of the health and safety staff and visitors, protection of vital infrastructure and other physical assets and restoration of the normal operations as quickly as possible. It also assists in proactively managing biological, chemical, physical, and radiological hazards in research and technical laboratories and services.

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