

# **XIII Annual Meeting**

**Center for Neurosciences and Cell Biology  
&  
Doctoral Programme in Biology and Experimental Biomedicine**

## **Abstracts Book**

**December 17-18, 2015  
FCTUC Auditorium, POLO II | Coimbra**



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# Programme

**December 17, 2015**

08.30h Registration

09.15h Opening Session:

Prof. Catarina Oliveira | Coordinator of CNC.IBILI Consortium

Prof. João Ramalho-Santos | President of CNC

09.45h **Special Session:** Helena Machado (*CES- Centre for Social Studies, University of Coimbra*) “Genomics, neurosciences and datasharing: sociological and ethical challenges”

## Session I – Neuroscience and Disease | *Chairs: Joana Marques & Elisabete Ferreira*

10.15h Sofia Ferreira | Role of P2 Receptors in the migration of medial ganglionic eminence-derived interneurons

10.25h Mariline Silva | Identification and characterization of a novel microRNA regulated by neuronal activity

10.35h Sofia Alçada-Morais | Adenosine A2A receptors control cortical neurons migration

10.45h António Carvalho da Silva | The Impact of Circadian Clock in the Neurobiology of Alzheimer’s Disease, Active vs Inactive Period in 3XTG-AD

10.55h *Coffee Break*

11.20h **Special Session:** Paula de Oliveira (*Center of Mathematics of the University of Coimbra*) “Mathematical models: paving the way for a personalized medicine”?

12.00h *Lunch*

## Session II – Metabolism aging and disease | *Chair: Sandra Amaral & Ermelindo Leal*

14.00h Susana Guerreiro | Decrease expression of BMP7 improve the angiogenesis in wound healing

14.10h Madalena Ribeiro | Insulin-induced cartilage degradation in osteoarthritis is associated to defective autophagy

14.20h José Santos Teixeira | Targeting dietary hydroxycinnamic acids to mitochondria: a refined strategy to improve nature

14.30h João Teodoro | The bile acid chenodeoxycholic acid directly modulates metabolic pathways in white adipose tissue in vitro: insight into how bile acids decrease obesity

## Session III – New preventive and therapeutic strategies | *Chairs: Rui Nobre & Chantal Fernandes*

14.50h Joana Duarte Neves | Neuropeptide Y mitigates neuropathology and motor deficits in mouse models of Machado-Joseph disease

15.00h Ana Maranhã | New mycobacterial targets for therapeutic intervention: function, structure and beyond

15.10h Patricia Pitrez | The vulnerability of smooth muscle cells derived from hutchinson-Gilford progeria IPSCS to arterial flow shear stress is mediated by metalloproteases

15.20h Lisa Rodrigues | Candida-host interaction: role of purines and adenosine A2A receptors

15.30h Sónia Pereira | Anti-inflammatory intestinal activity of an anthocyanin-rich fraction from portuguese blueberries in comparison to that of 5-Aminosalicylic acid, using colitis rat model

15.50: *Coffee Break*

16.20h **Special Session:** Luís Veríssimo | (*Manhattan Project & Faculty of Social and Human Sciences, NOVA University of Lisbon*) “Are you talking to me?”

## Session IV – Neuroscience and Disease | *Chairs: João Lopes & Mário Laço*

17.00h Ana Viegas | MicroRNA modulation targets amyloid- $\beta$  cascade in Alzheimer’s disease

17.10h Ana Plácido | Role of endoplasmatic reticulum stress in Alzheimer’s disease-associated endothelial dysfunction

17.20h Sandra Mota | Oxidative stress involving changes in NRF2 and ER stress in early stages of Alzheimer’s disease

# Programme

**December 18, 2015**

9.00h PhD movie 2

10.30h *Coffee-Break*

11.00h **Special Session:** Rui Travasso (*CFisUC, Universidade de Coimbra*) "Using mathematical modelling to understand sprout elongation in sprouting angiogenesis"

## Session V - Neuroscience and Disease | *Chairs: Sandra Santos & M<sup>a</sup> Joana Pinto*

11.30h Pedro Afonso | BDNF increases surface expression of GluN2B-containing NMDA receptors by a mechanism involving synaptic synthesis/activation of Pyk2

11.40h Sara Oliveira | Carbon monoxide as a metabolic and brain-blood barrier modulator with protective effects after stroke

11.50h Susana Sampaio | The synaptic proteome: a functional and quantitative study

12.00h Catarina Gomes | Adenosine A2A receptors control microglia remodelling, which parallel gender biases in developmental genesis and treatment of anxiety

12.20h **Special Session:** Paulo Peixoto (*ISR- Instituto de Sistemas e Robótica, Universidade de Coimbra*) "Robotics Meets The Bio World"

13.00h *Lunch*

## Session VI - Metabolism Aging and Disease | *Chairs: João Teodoro & Susana Guerreiro*

14.00h Ana Patrícia Marques | Dipeptidyl peptidase IV inhibition prevents adipocyte differentiation and ameliorates fibrosis in adipose tissue of obese mice

14.10h Paula Mota | Spermatogonial stem cell organization in felid testis as revealed by DBA lectin

14.20h Mafalda Bacalhau | Functional genomics analysis to gathering evidence for m.7486 mutation pathogenicity

## Session VII – Biotechnology | *Chairs: Ana Luisa Cardoso & Susana Rosa*

14.40h Sandra Jesus | Adjuvant nanocarriers for hepatitis B vaccine

14.50h Pedro Gouveia | Flexible nanofilms coated with aligned piezoelectric microfibers preserve the long-term contractility of cardiomyocytes

15.00h André Soares | RLR1 and RLR2, two novel Arabidopsis thaliana atypical aspartic proteases involved in primary root development and lateral root formation

15.15h *Coffee-Break*

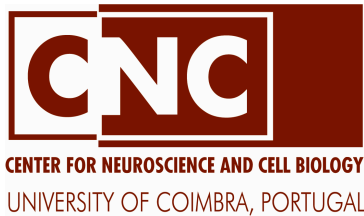
15.45h Poster Session

17.00h Marionet Theatre Performance "Atos de Laboratório" | (*Featuring CNC Researchers*)

17.30h Closing Remarks

20.30h *Dinner*

# Sponsors



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# **Abstracts for Oral Presentations**



## ROLE OF P2 RECEPTORS IN THE MIGRATION OF MEDIAL GANGLIONIC EMINENCE-DERIVED INTERNEURONS

S. Ferreira<sup>1,2</sup>, J.M. Marques<sup>1</sup>, S. Alçada-Morais<sup>1,2</sup>, R.A. Cunha<sup>1,3</sup>, R.J. Rodrigues<sup>1</sup>

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One of the fundamental processes for the establishment of a correctly wired cerebral cortical circuitry is neuronal migration, which is crucial to define the final allocation of neurons in the brain and the subsequent formation of synaptic networks. In fact, defects in the migration of cortical GABAergic interneurons have been associated with several neurological and psychiatric conditions (Marin, 2012, *Nat. Rev. Neurosci.* **17**, 107-120). Thus, unravelling the precise cellular and molecular mechanisms underlying the migration and positioning of interneurons during corticogenesis is of utmost importance.

Extracellular purines (ATP and adenosine) operate one of the most primitive receptor signalling systems, which suggests their putative involvement in development. Indeed, purinergic receptors (P1 and P2 receptors) have been identified in early embryonic stages, but their role in brain development remains ill defined. Our group recently demonstrated that the A2A receptors (A2AR) antagonism delays migration and the insertion of GABAergic neurons into the hippocampal circuitry (Silva *et al.*, 2013, *Sci Transl Med* **5**, 197ra104). Moreover, we could observe that P2 receptors bidirectionally modulate axonal outgrowth, as previously reported (del Puerto *et al.*, 2012, *J. Cell Sci.* **125**, 176-188), wherein P2Y1 receptors (P2Y1R) promote axonal elongation and the ionotropic P2X7 receptors (P2X7R) have the opposite effect. Interestingly, we detected immunoreactivity for both receptors at mid-late stage of embryogenesis (E11-onwards) coincident with the onset of interneuronal migration and in particular in medial ganglionic eminence (MGE)-derived interneurons (E13). Using single cell Ca<sup>2+</sup>-imaging we could confirm the functional expression of P2Y1Rs and P2X7Rs in those interneurons. Indeed, we now found that both are involved in medial ganglionic eminence (MGE)-derived interneurons migration. In MGE explants from E13-mice we observed that the selective blockade of P2Y1Rs with MRS2179 (10 µM) inhibited the migration of interneurons from the MGE explant. Conversely, we observed that the blockage of the ATP-gated P2X7R (BBG 100 nM), known to halt axonal elongation, promotes the migration of interneurons from the MGE explant.

Altogether, these results show that P2 receptors bidirectionally modulate MGE-derived interneurons migration, the inhibition mediated by P2X7R and the promotion of migration by P2Y1R. This re-enforces for a central role of purinergic signalling in neuronal migration and in corticogenesis.

## ADENOSINE A<sub>2A</sub> RECEPTORS CONTROL MICROGLIA REMODELLING, WHICH PARALLEL GENDER BIASES IN DEVELOPMENTAL GENESIS AND TREATMENT OF ANXIETY

L. Caetano<sup>1</sup>, H. Pinheiro<sup>2</sup>, P. Patrício<sup>3,4</sup>, C. Cunha<sup>4</sup>, A. Mateus-Pinheiro<sup>3,4</sup>, N. D. Alves<sup>3,4</sup>, F. Baptista<sup>2</sup>, S. Henriques<sup>2</sup>, A. R. Santos<sup>4</sup>, S. G. Ferreira<sup>1</sup>, V. Sardinha<sup>4</sup>, J. F. Oliveira<sup>4</sup>, N. Sousa<sup>3,4</sup>, A. F. Ambrósio<sup>2,5</sup>, R. A. Cunha<sup>1,2,5</sup>, A. J. Rodrigues<sup>3,4</sup>, L. Pinto<sup>3,4</sup>, C. A. Gomes<sup>1,2,5</sup>

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Developmental risk factors, such as the exposure to stress or high levels of glucocorticoids (GC), may contribute to the pathogenesis of anxiety disorders. GC, although provided with therapeutic benefits, are key mediators of stress responses, detrimental to the developing brain (“programming” effects). The immunomodulatory role of GC and the immunological fingerprint found in animals prenatally exposed to GC point towards an interplay between the immune and the nervous systems in the etiology of these disorders. Microglia are immune cells of the central nervous system affected by stress-related conditions, namely anxiety. Adenosine A<sub>2A</sub>R, as regulators of microglia and involved in the pathophysiology of anxiety, emerge as molecular candidates in mediating the “programming effects” of GC. In the current study we performed a comparative analysis in males and females prenatally exposed to the GC dexamethasone (DEX) and evaluated the impact of this manipulation on the adenosinergic system, microglia morphology, behaviour and corticosterone levels. We report that prenatal exposure to GC triggers changes in the adenosinergic A<sub>2A</sub>R system that are gender-specific, long-lasting and paralleled by a profound remodelling of microglial cell processes in the prefrontal cortex (PFC). Microglial cells re-organization responds in a gender-specific manner to the chronic treatment with a selective A<sub>2A</sub>R antagonist, which was able to ameliorate microglial processes abnormalities and anxiety behaviour in males, but not in females.

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Neuronal migration is a fundamental process during brain development essential for the formation of brain citoarchitecture and the establishment of neuronal/synaptic networks. Indeed, impairment in neuronal migration is one of the major causes of cortical malformation, which has been shown to be associated to several neurological and psychiatric disorders (Neuron 60:273-84). Hence, it is of upmost importance to unravel the mechanisms driving neuronal migration. In this regard, recent studies demonstrate that adenosine type 2A receptor (A2AR) for adenosine controls interneurons migration (Sci. Trans. Med. 5:197ra104). We now aimed to evaluate if A2AR is also involved in the migration of cortical principal neurons. For that purpose we first evaluated the impact of the genetic deletion (A2AR KO) or the pharmacological blockade of A2AR on cortical neurons migration during embryonic development.

In comparison to their wild-type littermates, embryos lacking the A2AR showed a delayed migration of cortical principal neurons at embryonic day 17 (E17). Similarly, embryos exposed to the A2AR antagonist SCH58261 (daily 0.1mg/kg i.p. injection in pregnant females from E13 to E16) have shown delayed migration when compared to with embryos exposed vehicle. These effects should be due to A2ARs expressed by migratory neurons, identified by immunohistochemistry, since *in utero* electroporation of plasmid encoding shRNA specific for A2AR also delays migration at the same developmental stage (E14-E17). The neuronal migration delay observed either in A2ARKO mice embryos or upon the knockdown (shRNA) or the pharmacological blockade of A2ARs occurs mostly in the intermediate zone, where it was observed an accumulation of neurons. It is well-known that it is required a transition from a multipolar to a bipolar shape at the intermediate zone and the establishment of an axon-like leading process in order to the neurons to proceed their migration into the cortical plate (Nat. Neurosci. 12:1693-700). Interestingly, we found in rat hippocampal neurons that the pharmacological activation of A2AR with the selective agonist CGS21680 (30 nM) from days in vitro (DIV) 1 onwards, leads to the formation of aberrant secondary axons (SMI-31 positive neurites) and a correlated reduction in the number of dendrites (MAP2-positive neurites) in hippocampal neurons at DIV3. Moreover, we have also gather evidences that this should involve the regulation of CRMP2 protein, a microtubule-associated protein highly expressed in the developing nervous system crucial for axon specification and outgrowth (Cell 120:137-49).

We found that the pharmacological activation of A2AR with CGS21680 (30nM) increased the inhibitory phosphorylation of GSK3 $\beta$ S9 and concomitantly decreased the phosphorylation levels of CRMP2 at Thr514 (GSK3 $\beta$  target), which promotes its interaction with tubulin (Nat. Cell Biol. 4:583-91) and consequently the formation and outgrowth of axons.

Altogether, these results show that A2AR is required for proper cortical principal neuronal migration, in particular in the transition from the intermediate zone into the cortical plate most likely by contributing to the establishment of neuronal polarity through the de-repression of CRMP2 activity

(Support: EXPL/NEU-NMC/0671/2012, PEst-C/SAU/LA0001/2013-2014 and SFRH/BD/51963/2012)

## THE IMPACT OF CIRCADIAN CLOCK IN THE NEUROBIOLOGY OF ALZHEIMER'S DISEASE, ACTIVE VS INACTIVE PERIOD IN 3XTG-AD

António M. Carvalho da Silva<sup>1</sup>, C. Lemos<sup>1</sup>, John Jones<sup>1</sup>, Ana Cristina Rego<sup>1,2</sup>, Rodrigo A. Cunha<sup>1,2</sup>

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Alzheimer's disease (AD) is a brain disease which affects mainly older people. It's progression leads to impaired memory, destroyed motor skills and can eventually cause death. Despite recent advances in molecular biology and genetics, the etiology of AD is still undefined.

AD can be considered as a disease of aging. Aging has been related to a progressive decline in cognitive functions. The circadian system also suffers from age-related decline in the central circadian clock, which impacts the amplitude of rhythmic behaviors and fragmentation of sleep patterns, commonly associated with aging in humans and especially relevant in AD patients.

Importantly, circadian disturbances have been reported early in AD progression. The triple transgenic (3xTg-AD) mouse model of AD displays abnormalities in circadian rhythmicity prior to AD pathology, making this mouse model instrumental to investigate the interplay between circadian clock, metabolism and the link to AD pathogenesis. This study evaluated the impact of the circadian profile on cognitive performance in 24 weeks old 3xTg-AD mice *versus* age-matched Control (Ctrl) mice, explored how this correlated with hippocampal long-term potentiation and how mitochondrial function an indicator of brain metabolism is impacted by circadian biology.

The diurnal variation in hippocampus-dependent learning tasks performance of 3xTg-AD and Ctrl animals was assayed. The MWM results show that independent of the time of day cognitive deficits were observed in 3xTg-AD, a correlation between Zt's and genotypes was only possible, when making use of the Spatial Reverse learning task. These results suggest that the pattern of circadian variation of memory performance depends on the type of task and the impact of circadian clock on AD pathology is better outlined in more complex tasks.

Regarding the amplitude of hippocampal long term potentiation (LTP) an electrophysiological correlate of memory related processes: electrical signals recorded extracellularly at Schaffer fibers/CA1 pyramid synapses, exhibited a circadian profile of LTP induction in WT animals, which was not observed in the 3xTg-AD.

Disturbances in mitochondrial functions play a part in impaired neuroplasticity., also mitochondrial dysfunction and oxidative stress have been implicated in the pathophysiology of AD. Therefore mitochondrial membrane potential ( $\Delta\Psi_m$ ) was measured in P2 crude cortical synaptosomes using the fluorometric probe TMRM+, the susceptibility to oxidative stress upon exposure to Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) revealed a greater depolarization at Zt16 which correlates with a greater susceptibility to damage depending on the Zt. Simultaneously, intracellular calcium ( $[Ca]$ ) levels were measured using the fluorimetric probe Fura2-AM; this study revealed a inability to maintain  $[Ca]$  levels by 3xTg-AD at Zt16; H<sub>2</sub>O<sub>2</sub> stimulus didn't reveal significative changes between Zt's and genotypes.

This study demonstrates the impact of the circadian rhythm on key traits of AD pathogenesis. Outlines the prevalence of diurnal variation at different levels and pinpoints the need to better characterize the neurobiology of disease and integration of circadian rhythms as a crucial variable.

## DECREASE EXPRESSION OF BMP7 IMPROVE THE ANGIOGENESIS IN WOUND HEALING

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(4) The Portuguese Diabetes Association (APDP), Lisbon, Portugal

*\*This authors contributed equally to the work.*

**BACKGROUND:** Diabetic foot ulcer remains one of the most serious secondary complications in diabetic patients with an impact not only for them, but also in health care costs. Neuropathy,

ischemia, and vascular diseases affect the development of ulcer. Deregulation levels of inflammatory factors, growth factors and proteins of the family of transforming growth factor  $\beta$  (TGF- $\beta$ ) as the bone morphogenetic protein 7 (BMP7) has been associated with the difficulty in wound healing in diabetes. The balance between these two molecular signaling pathway TGF- $\beta$  / BMP7 may be a new therapeutic target for wound healing in diabetic patients. This study evaluated the role of BMP7 in wound healing in the skin of type 1 diabetic and non-diabetics mice.

**METHODS:** Diabetes was induced by streptozotocin in controls and transgenic mice that partially express the BMP7 gene (BMP7 +/- ). The transcription of BMP7 mRNA and TGF $\beta$ 1 mRNA by qRT-PCR was analyzed at day 0 and 10, post-wounding, using skin biopsies. Through histological analysis (H&E and Masson's Trichrome) the organization of the extracellular matrix, inflammation and collagen deposition was also evaluated. The presence of blood vessels in tissue, was evaluated with CD31+ staining by immunohistochemistry.

**RESULTS:** There was no statistically significant difference in wound healing between the non-diabetic heterozygous BMP7 mice (BMP7 +/- ) and non-diabetic wild type mice (WT). In the presence of diabetes, the BMP7 +/- mice (DM BMP7 +/- ) showed a significantly decreased healing capacity compared to diabetic WT mice, at day 10. At day 0, non-diabetic BMP7 +/- mice showed a decrease in BMP7 gene expression compared to non-diabetic WT mice. In the presence of diabetes at day 10 post-wounding, the expression of BMP7 was significantly higher in BMP7 +/- mice (DM BMP7 +/- ) compared to diabetic WT mice (DM WT). Moreover, at day 0 the expression of TGF- $\beta$  mRNA was significantly higher in the diabetic BMP7 +/- compared to nondiabetic BMP7 +/- and diabetic WT mice, while at day 10, there were no statistically significant differences between groups. In addition, the extracellular matrix of the skin, at day 0 and day 10, was better organized in the WT mice (diabetic and non-diabetic) compared to BMP7 +/- mice (diabetic and non-diabetic). The same was observed regarding the inflammatory process. At day 0, the collagen deposition was higher in non-diabetic mice (both in WT and BMP7 +/- ) compared to diabetic mice (both in WT and BMP7 +/- ). At day 10, diabetic BMP7 +/- mice had more collagen deposition compared to diabetic WT mice. The number of the skin blood vessels was higher in non-diabetic BMP7 +/- mice compared to non-diabetic WT mice, at day 0.

**CONCLUSION:** This study revealed that the decrease in the levels of BMP7 can influence skin wound healing due to increased collagen deposition and alterations in angiogenesis.

**Grant acknowledgments:** This study was supported by FCT, COMPETE-FEDER and POPH/FSE, through the following grants/strategic funding: UID/NEU/04539/2013 (CNC.IBILI), EXPL/BIM-MED/0492/2012, SFRH/BPD/88745/2012.

# INSULIN-INDUCED CARTILAGE DEGRADATION IN OSTEOARTHRITIS IS ASSOCIATED TO DEFECTIVE AUTOPHAGY

Madalena Ribeiro<sup>1,2</sup>, Paloma López de Figueroa<sup>1</sup>, Francisco J. Blanco<sup>1</sup>, Alexandrina F. Mendes<sup>2</sup>, Beatriz Caramés<sup>1</sup>

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**Background:** Autophagy, a key cellular quality control mechanism, is defective in Osteoarthritis (OA) and Type 2 Diabetes (T2D). T2D has been proposed as a risk factor for OA. Although epidemiological studies suggest a strong association between these diseases, how T2D may have an effect on the deterioration of articular cartilage is still unknown. *The objective of this study is to understand the role of autophagy in the articular cartilage function under diabetic conditions.*

**Methods:** Human chondrocyte cell line (TC28a2) and primary human chondrocytes (HC) were cultivated in DMEM high glucose (25 mM) and treated with Insulin (10, 100, 500 nM) for 2, 6 and 24hours. Activity of LC3-II, Akt and pS6 was evaluated by Western blotting (WB). To investigate whether autophagy activation protects from diabetic conditions, autophagy was induced by Rapamycin (10  $\mu$ M). Human cartilage explants were cultivated in DMEM 25mM glucose and insulin (100, 500, 1000nM) for 24hours to evaluate histopathological changes. MMP-13 and IL-1 $\beta$  expression was determined by immunohistochemistry and WB, respectively. Expression of LC3 and p-rpS6 was determined by WB in human chondrocytes from Non Diabetic-OA and Diabetic-OA patients.

**Results:** In the presence of high glucose and increased doses of insulin autophagy was decreased in a dose dependent-manner in human chondrocytes, as indicated by LC3II expression, the main marker of autophagy activation (TC28-a2;  $p < 0.05$  at 6hours post-treatment; HC;  $p < 0.01$  at 24hours post-treatment). To investigate the mechanism by which autophagy is reduced by insulin, Akt and pS6 phosphorylation was analyzed. We observed a significant increase in p-Akt and p-rpS6 activity, suggesting that insulin effect is mediated by Akt/mTOR pathway (TC28-a2  $p < 0.05$  at 6hours; HC;  $p < 0.01$  at 2hours). Autophagy activation by Rapamycin reversed insulin effects on LC3 and p-rbS6 expression (Tc28a2 and HC:  $p < 0.05$ ), indicating that autophagy induction prevents insulin-mediated autophagy signaling downregulation. To evaluate the impact of insulin-mediated autophagy regulation in the context of articular cartilage biology, cartilage explants were treated with insulin (100, 500 and 1000 nM) for 24hours. Histological analysis indicated a loss of proteoglycans and increased MMP-13 and IL-1 $\beta$  expression ( $p < 0.01$ ) after insulin treatment. Remarkably, chondrocytes from OA-diabetic patients showed decreased LC3 and increased p-rpS6 expression compared to Non-Diabetic OA patients.

**Conclusions:** Our findings demonstrate that diabetic conditions decrease autophagy by an Akt/mTOR dependent mechanism. Pharmacological activation of autophagy might protect against T2D in human chondrocytes. Our data also indicate that chondrocytes from OA-diabetic patients exhibit a deficient autophagy. Taking together, these results suggest that impaired autophagy might be one of the mechanisms by which T2D diabetes accelerates cartilage degradation.

## TARGETING DIETARY HYDROXYCINNAMIC ACIDS TO MITOCHONDRIA: A REFINED STRATEGY TO IMPROVE NATURE

José Teixeira<sup>1,2,3</sup>, Farhan Basit<sup>3</sup>, Paulo J. Oliveira<sup>2</sup>, Peter H.G.M Willems<sup>3</sup>, Jori Joosten-Wagenaars<sup>3</sup>, Fernando Cagide<sup>1</sup>, Sofia Benfeito<sup>1</sup>, Catarina Oliveira<sup>1</sup>, Fernanda Borges<sup>1</sup>, Werner J.H. Koopman<sup>3</sup>

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Polyphenols such as hydroxycinnamic (HCA) have been extensively studied due to their antioxidant properties, which present different mechanisms at several subcellular compartments. In addition, modulation of mitochondrial biogenesis, electron transport chain complex activity and ATP synthesis are particularly important mechanisms of action of polyphenols, which go beyond their recognized antioxidant properties. At the dosages normally consumed by humans in diet, phytochemicals are prime inducers of adaptative cellular stress responses pathways, contributing for their beneficial effects. HCA can be oxidized to a quinone form, which may directly or indirectly act as an electrophile, acting as potent regulators of the cellular redox status. Therefore, these hormetic-like effects of phytochemicals can be a promising strategy to improve the cellular antioxidant status and contribute to decrease oxidative stress. Mitochondria adapt to environmental factors and respond to energetic cues by producing effectors that activate multiple pathways related to oxidative stress balance. Although at higher doses ROS unquestionably exert detrimental effect on cellular integrity, transient ROS signaling initiates molecular stress responses that modulate gene expression and results in a cellular response that contributes for increased resistance to stress.

In order to direct HCA to mitochondria for locally-mediated effects, we recently designed and synthesized a series of selectively-targeted agents, namely AntiOxCIN<sub>4</sub> and AntiOxCIN<sub>6</sub>. Although treatment with these two novel molecules increased intracellular ROS in human fibroblasts, this was associated neither with cell death nor with mitochondrial alterations on  $\Delta\Psi$  and morphology. Instead, intracellular ROS may play an important role in the regulation of intracellular protective pathways. In fact, AntiOxCIN<sub>4</sub> increased GSH content and MnSOD in 45%. Up-regulation of intracellular antioxidant defense system by AntiOxCIN<sub>4</sub> slightly protected cells against subsequent stress-inducing events. Despite mitochondria-targeted antioxidants increased mitochondrial NAD(P)H autofluorescence, which may confer more reducing power to glutathione reductase (GR), AntiOxCIN<sub>4</sub> did not prevent or reverted BSO-induced cell death. The data suggest that AntiOxCIN<sub>4</sub> may play a role in the maintenance of intracellular GSH homeostasis by increasing its supply through stimulation of GSH redox cycling and GR activity.

Our data is a further step in the development of mitochondriotropic antioxidants based on dietary scaffolds that possess hormetic-like effect, which can be an indirect mechanism to exert their antioxidant activity. This new type of antioxidants can be used as therapeutic agents in the treatment of oxidative stress-related diseases.

**Acknowledgements:** This work was supported by the Foundation for Science and Technology (FCT), Portugal PTDC/DTP-FTO/2433/2014 funded by FEDER/COMPETE/O.E. funds and QREN (FCUP-CIQ-UP-NORTE-07-0124-FEDER-000065) J. Teixeira (SFRH/BD/79658/2011), C. Oliveira (SFRH/BD/88773/2012), S. Benfeito (SFRH/BD/99189/2013) and F. Cagide (SFRH/BPD/74491/2010) are supported by FCT Doctoral and Postdoctoral grants.

## THE BILE ACID CHENODEOXYCHOLIC ACID DIRECTLY MODULATES METABOLIC PATHWAYS IN WHITE ADIPOSE TISSUE *IN VITRO*: INSIGHT INTO HOW BILE ACIDS DECREASE OBESITY

João Soeiro Teodoro, Anabela Pinto Rolo, Filipe Valente Duarte, Carlos Marques Palmeira and Rui Albuquerque Carvalho

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Obesity and associated conditions have reached epidemic levels throughout the world. Current pharmacological strategies have failed to be efficient in reducing circulating metabolites' levels, decreasing adiposity and improving health. To this goal, a novel strategy has emerged in the potential therapeutic use of bile acids (BA) to fight obesity.

In recent years, BA have been found to have a hypoglycemic and hypolipidemic activity in both *in vivo* animal models and obese patients (Teodoro et al., 2011; Teodoro et al., 2014). BA are currently thought to have two major receptors that mediate these functions, the Farnesoid X Receptor (FXR) and the G protein-coupled receptor 5 (TGR5). Both have very different and sometimes contradicting effects, depending on type of BA used and target tissue type. Regardless, systemic BA exposure causes a total reversal of obesity and diabetic statuses, an effect at least in part caused by enhanced brown adipose tissue (BAT) thermogenesis (Teodoro et al., 2014; Watanabe et al., 2006). This is particularly interesting due to the recent discovery of functional BAT in adult humans (Ouellet et al., 2012). However, the lack of consensus about route of action and key target modulators of metabolic pathways involved in BA activity, coupled with the inherent BA cytotoxicity has rendered the development of more safe and potent BA-effect mimetic pharmacological agents difficult. As such, we report here that the BA chenodeoxycholic acid (CDCA) is a powerful reducer of *in vitro* adipocytical obesity. Furthermore, NMR analysis demonstrates that CDCA is capable of inducing an acceleration of several metabolic pathways, including the citric acid cycle, mitochondrial oxidative phosphorylation, lipidic oxidation and a substrate-futile, energy depleting, triglyceride/glycerol+free fatty acid cycle.

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## NEUROPEPTIDE Y MITIGATES NEUROPATHOLOGY AND MOTOR DEFICITS IN MOUSE MODELS OF MACHADO-JOSEPH DISEASE

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Machado-Joseph disease (MJD) is a dominantly-inherited neurodegenerative disorder associated with an expanded polyglutamine tract within the protein ataxin-3. This mutated protein causes progressive impairment of motor coordination, associated to the neurodegeneration of cerebellum, pons, brain stem, *substantia nigra* and striatum. The currently available therapies do not allow modification of disease progression. In the present work we investigated the impact of neuropeptide Y (NPY) on neurochemical and behavioural modifications in rodent models of MJD since: i) NPY and Y receptors are abundant and widely distributed in the CNS, ii) NPY has its levels altered in different brain disorders; and iii) NPY exerts neuroprotective effects by multiple pathways associated with MJD mechanisms of disease.

We analysed whether NPY levels are altered in post mortem patient brain tissue and animal models of MJD. Additionally, we evaluated the impact of NPY overexpression in the striatum and cerebellum of a lentiviral-based model and a transgenic model of MJD, respectively, through stereotaxic injection of adeno-associated viral vectors (AAVs) encoding either NPY, or EGFP as control. Lentiviral-mediated MJD animals were sacrificed at 4 and 8 weeks post-surgeries for immunohistochemical and western blot analysis of mutant ataxin-3 inclusions and loss of DARPP-32 staining. Motor behaviour defects of MJD transgenic animals were evaluated by stationary rotarod, beam walking and footprint patterns, before and 4 and 8 weeks after AAV-injections.

NPY mRNA levels of brain extracts of both post mortem patient brain tissue (dentate nucleus, n=2) and mice (striatum of lentiviral-mediated overexpressing mutant-atx3 animals, and dissected cerebella of transgenic MJD, n=3/5) were significantly decreased. Additionally, we observed a significant decrease (n=5) in the number of NPY-positive interneurons upon striatal overexpression of mutant atx3 in the lentiviral-based MJD model, justifying the reinstatement of NPY levels.

NPY overexpression in the striatum of the MJD lentiviral model mediated a significant decrease (n=4) in the number of mutant ataxin-3 inclusions and a 55 ± 9% reduction of the striatal lesion assessed by DARPP-32 immunoreactivity. In addition, NPY overexpression in the cerebella of transgenic MJD mice mediated significant: i) reduction in the latency to fall of the rod (n=9-13); ii) improvement to cross the round beams (n=9-13); and iii) almost close to complete rescue of footprint overlap (n=6-8).

Taken together, these data show that NPY is reduced in MJD and that NPY overexpression reduces MJD-associated neuropathology and motor-related deficits, which supports NPY overexpression as a candidate strategy to modulate and prevent the abnormal neurochemical and motor changes in MJD.

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## NEW MYCOBACTERIAL TARGETS FOR THERAPEUTIC INTERVENTION: FUNCTION, STRUCTURE AND BEYOND

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The World Health Organization alerts on an imminent pandemic Tuberculosis-Diabetes. The emerging *Mycobacterium tuberculosis* strains resistant to our dated antibiotic arsenal and the lessons from the HIV-AIDS pandemic, all merge into a health priority urging identification of new metabolic pathways that may grant new targets against which new drugs can be created<sup>[1,2]</sup>.

Mycobacteria assemble a distinctive lipid-rich cell envelope to which they owe much of their pathogenicity. They also rely on unique intracellular polymethylated polysaccharides (PMPs) to modulate the synthesis of fatty acids, precursors of cell wall lipids. The 6-O-methylglucose lipopolysaccharides (MGLPs), identified half-century ago, are branched and partially acylated polymers of methylglucose, with an invariable octanoate attached to the MGLP reducing end glycerate<sup>[3]</sup>. Notwithstanding its vital role, MGLP biosynthetic enzymes remained obscure until recently. We have identified key genes of this pathway, most of which proposed to be essential for *M. tuberculosis* growth, and characterized the corresponding enzymes for their potential as drug targets<sup>[4]</sup>. A novel pathway discovered recently in *M. tuberculosis* involves the enzymes trehalose synthase (TreS), maltokinase (Mak) and a maltosyltransferase (GlgE) and channels trehalose through maltose and maltose-1-phosphate to glycogen extension which is later branched by GlgB<sup>[5]</sup>. Since glycogen and MGLP share the same basic architecture, the TreS-Mak-GlgE pathway was considered an alternative route for extension of MGLP.

To lay experimental foundations for drug discovery, we sought determination of these enzymes' 3D structures, all proposed to be essential for *M. tuberculosis* growth<sup>[6]</sup>. We revealed the first high-resolution structure of GlgE and crucial interactions between domains of opposing monomers with implications in catalysis<sup>[7]</sup>. Herein, we describe unique structural features of Mak uncovering a new family of eukaryotic-like kinases<sup>[8]</sup>. Furthermore, we have identified a rare and essential octanoyltransferase (OctT) in *M. tuberculosis* that transfers octanoate to the MGLP earliest intermediates. Combined enzymatic, synthetic chemistry, NMR spectroscopy and mass spectrometry approaches suggest that, in contrast to a prevailing consensus, octanoate is not attached to glycerate but to the 1<sup>st</sup> glucose of MGLP's main chain, raising questions about its *in vivo* architecture and physiological role<sup>[9]</sup>. Our results add new insights into an intricate and vital pathway of mycobacterial glycobiology and crucial scaffolds for drug discovery, placing us closer to devise better strategies to face the looming new challenges posed by an old disease.

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# THE VULNERABILITY OF SMOOTH MUSCLE CELLS DERIVED FROM HUTCHINSON-GILFORD PROGERIA SYNDROME (HGPS) IPSCS TO ARTERIAL FLOW SHEAR STRESS IS MEDIATED BY METALLOPROTEASES

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## Introduction

HGPS is caused by a single mutation of the lamin A gene (LMNA) resulting in the generation of an abnormal lamin A named “progerin”. Progerin lacks the proteolytic cleavage site normally used to remove the farnesylated carboxy terminus from lamin A during posttranslational processing [1]. Progerin accumulates with successive cell passage number, leading to progressive nuclear envelope deformations and invaginations, and premature senescence. In general, patients die because of myocardial infarction or stroke [2]. In the last years, pre-clinical [3] and clinical treatments [4, 5] have been proposed for HGPS patients; however, further efforts are needed in the design of more effective therapies.

One of the hallmarks of the disease is the loss of SMCs in the medial layer of large arteries with replacement by collagen and extracellular matrix, and in many cases calcification [6, 7]. Studies in transgenic mice carrying the G608G mutated human lamin A showed progressive loss of SMCs, elastic fiber breakage, thickening of the adventitia and medial layer, accumulation of hyaluronan and collagen [8]. Although lamin A and progerin were expressed in several tissues in the transgenic mice, progeria phenotype is essentially observed in SMCs. This agrees with the fact that children with HGPS die because of progressive arterial occlusion. The mechanism underlying the vulnerability of the SMCs to arterial flow shear stress remains poorly understood, in part due to the difficulty of having SMCs from HGPS patients due to their rarity and fragile condition. Induced pluripotent stem cells (iPSCs) offer an unlimited source of SMCs to study the HGPS disease and particularly the vulnerability of SMCs to arterial flow shear stress.

## Results and Discussion

SMCs derived from HGPS-iPSCs share similar features observed in progerin-expressing cells. When HGPS-SMCs were cultured under arterial flow conditions (20 dynes/cm<sup>2</sup>) they show an up-regulation of progerin and osteogenic markers followed by their detachment from the culture substrate.

It has been shown that wild-type mouse *Lmna* gene with a mutant allele that carried the c.1827C>T;p.Gly609Gly mutation recapitulate most of the described alterations associated with HGPS, including the loss of vSMC. Take this into account we isolated SMC from wild-type mice (WT mSMC) and homozygous *Lmna* G609G/G609G (HOZ mSMC). To confirm the human HGPS-SMC results we cultured mSMC under flow shear stress conditions (120 dyne/cm<sup>2</sup>) and it was verified that WT mSMC were able to be cultured under flow conditions up to 26 days without loss of cells. On the other hand, HOZ mSMC detached from the substrate after 8/9 days. These results confirm that mSMC are vulnerable to flow shear stress and are in agreement with HGPS-iPSC SMC results.

Microarray analysis comparing HGPS-SMCs cultured in static conditions and under slow conditions reveals that HGPS-SMCs have significant changes in extracellular matrix (ECM) secretion and MMP expression. Moreover, HGPS-SMC detachment is prevented by the inhibition of MMPx. The administration of a MMP inhibitor in HGPS mice also prevents the loss of vascular SMC on aortic arch media.

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## CANDIDA-HOST INTERACTION: ROLE OF PURINES AND ADENOSINE A<sub>2A</sub> RECEPTORS

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*Candida* spp., members of the human body normal flora, can turn into aggressive agents of opportunistic infections, namely in individuals with immune diseases or in debilitated conditions. This is most frequent in elderly individuals, where the natural immunosenescence upon aging dampens the ability to control infections, including those caused by fungi. Yeast recognition and clearance by host phagocytic cells, such as macrophages, is a complex procedure involving multiple recognition systems and inflammatory responses. Purines, namely extracellular ATP and adenosine, operate an important role in immunity and inflammation homeostasis. In particular, the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) critically contributes to the fine-tuning of inflammatory and immune responses, prompting an efficient elimination of threats while minimizing tissue damage.

This work aimed to explore the hypothesis that adenosine and its sensing devices may constitute one of the systems exploited by *Candida* spp., in particular by *Candida albicans*, to modulate the response of macrophages, bolstering its pathogenic success. This study focused on the role of A<sub>2A</sub>R to control the efficiency of macrophage phagolysosomes to clear yeasts. It was observed that yeast infection failed to increase of A<sub>2A</sub>R gene expression, in contrast to the increased gene expression upon exposure of macrophages to lipopolysaccharide (LPS) from Gram negative bacteria. Furthermore, upon yeast infection, A<sub>2A</sub>R were localized around phagosomes containing yeasts cells. The use of pharmacological approaches, with both A<sub>2A</sub>R agonists and antagonists, as well as peritoneal macrophages from A<sub>2A</sub>R knockout mice, allowed concluding that A<sub>2A</sub>R controlled the phagocytic efficiency of macrophage to internalize and clear *C. albicans* cells. Moreover, during the course of infection with *C. albicans* there was a decrease of the extracellular levels of ATP, a danger signal contributing to stimulate inflammation leading to clearance of pathogens, thus forcing a lower efficiency of macrophages. Furthermore, *C. albicans* ecto-nucleotidases and ecto-phosphatases, enzymes involved in extracellular adenosine formation, revealed some special properties relevant to its pathogenesis and survival inside the phagolysosome. Finally, an *in vivo* model of sustained *C. albicans*-gastrointestinal infection was constructed aiming to define the involvement of A<sub>2A</sub>R on the susceptibility to yeast infection and on the inflammatory response of gastrointestinal tissues from young, adult and aged mice; this allowed establishing a correlation between infection, inflammation, ageing and A<sub>2A</sub>R localization in the gastrointestinal tract.

Overall, the present work shows that upon interaction with macrophages, *Candida* is able to change immune and inflammatory responses by modulating both the source of adenosine activating A<sub>2A</sub>R and by re-locating A<sub>2A</sub>R: this favors the silencing of macrophages responses and is proposed to constitute a novel strategy contributing to the success of *Candida* spp. as pathogens as well as for the persistence of endogenous reservoirs of opportunistic agents of infection.

\* Part of LR PhD thesis; work included in the manuscripts:

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## ANTI-INFLAMMATORY INTESTINAL ACTIVITY OF AN ANTHOCYANIN-RICH FRACTION FROM PORTUGUESE BLUEBERRIES (*VACCINIUM CORYMBOSUM* L.), IN COMPARISON TO THAT OF 5-AMINOSALICYLIC ACID, USING A COLITIS RAT MODEL

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Inflammatory bowel disease (IBD) is a chronic inflammatory disorder characterized by periods of remission and relapses, whose etiology still needs to be clarified. This disease does not have cure yet and among the therapeutic strategies, 5-aminosalicylic acid (5-ASA) is a well-established anti-inflammatory drug. Unfortunately, it is not devoid of adverse effects, thus requiring new alternative therapeutic strategies. In this context, anthocyanins have been a focus of intensive research, as they are the most common dietary polyphenols, due to their antioxidant activity and ability to modulate inflammatory pathways. Its poor intestinal absorption, associated with its high intake makes the gastrointestinal tract the compartment where the concentration of these compounds achieves the highest value, which may play an important role in modulating IBD.

This way, the aim of this work was to assess the intestinal anti-inflammatory action of an anthocyanin-rich fraction (ARF) isolated from Portuguese blueberries (*Vaccinium corymbosum* L.) in a Wistar rat model of colitis, and compare its efficacy to that of 5-ASA.

The isolated ARF showed a very high content and diversity of anthocyanins, as studied by HPLC-DAD analysis, with malvidin-3-galactoside and petunidin-3-arabinoside in the highest concentrations. Its anti-inflammatory efficacy was evaluated in a well characterized 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced rat model of colitis, after daily administration of ARF by intragastric infusion (10 mg anthocyanin/kg/day), during 8 days, as compared with 5-ASA (100 mg/kg/day). This treatment significantly counteracted the TNBS-induced inflammatory markers, namely: i) body weight loss and colonic tissue damage, in terms of damage extension, adhesions and colonic weight/length ratio; ii) leukocyte infiltration, as measured by myeloperoxidase activity and its expression and alkaline phosphatase activity in colon homogenates; and iii) inflammatory enzymes, as measured by iNOS and COX-2 expressions. Additionally, the ARF treatment was also able to modulate the intestinal antioxidant defences, by increasing the GSH/GSSG ratio and GPX activity. These results, in comparison to those with 5-ASA treatment, indicate a better efficacy of ARF against the inflammatory process. In conclusion, blueberry anthocyanins show a significant intestinal anti-inflammatory action, which seems to be due to a combination of action mechanisms: modulation of the altered immune response, anti-oxidant activity, and downregulation of inflammatory signalling pathways. Taken together, these data indicate that the anthocyanin fraction counteracts colitis severity and gather conditions to a faster recovery, suggesting its potential role in IBD management.

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## MICRORNA MODULATION TARGETS AMYLOID- $\beta$ CASCADE IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most common form of dementia worldwide, characterized by progressive neuronal loss and neuroinflammation. Although two hallmark lesions can be highlighted: senile plaques, composed by the amyloid- $\beta$  peptide (A $\beta$ ) and neurofibrillary tangles, containing hyperphosphorylated tau protein, the exact molecular mechanisms underlying AD remain unclear.

Several studies have demonstrated that miRNA expression alters during senescence, contributing to modification of gene expression patterns. In this context, the identification of deregulated miRNAs and the correction of their levels in brain of Alzheimer's disease patients and animal models may contribute to the development of new therapies for AD.

In this study we employed bioinformatic tools to select miRNAs with high affinity to the 3'UTR region of APP and BACE1 genes and the identified binding sites were biochemically validated through the luciferase assay. Additionally, a decrease in APP and BACE1 mRNA levels, consistent with an effective miRNA/mRNA pairing, was observed upon transfection of cultured human and mouse neuronal cell lines with miRNA mimics or lentiviral constructs containing the selected miRNA sequences.

Based on these results, we selected one miRNA, able to simultaneously modulate APP and BACE1, to perform *in vivo* studies in the 3xTg-AD animal model. For this purpose, lentiviral particles, encoding the precursor form of this miRNA, were delivered through stereotactic injection bilaterally into the mice hippocampus.

Different behavioral tests were performed before (12 months) and after (16 months) lentiviral delivery, in order to analyze learning and memory differences between four animal groups: 3xTg-AD untreated (n=8), 3xTg-AD injected with a negative control lentivirus (n=5); 3xTg-AD treated with the miRNA lentivirus (n=7); and aged-matched wild-type mice (non-transgenics, n=8). Working memory was accessed through the spontaneous alternation paradigm in the T-maze test and spatial learning and memory were analyzed using the Barnes maze test. Results showed a significant improvement in cognitive function in animals treated with the miRNA lentivirus, with respect to the untreated group and the negative control group, particularly in what concerns T-maze performance.

Additionally, histological studies have shown that animals injected with the miRNA of interest present a strong reduction in the number of plaques in the subiculum and hippocampus, as well as a reduction in the number of neurons staining positive to human APP, compared to control groups. These results suggest that miRNA overexpression is able to reduce the number and size of A $\beta$  deposits and also to decrease human APP levels *in vivo*, in the 3xTg-AD mouse model.

Our study demonstrate that miRNA modulation is a promising strategy to decrease the *in vivo* levels of APP and BACE1 genes, reduce A $\beta$  deposition, and ameliorate the cognitive function of 3xTg-AD animals. Given the high conservation of the selected miRNA and its binding site across species, it is likely that new significant insights into the ageing process may arise from this specific miRNA modulation, supporting new diagnostic and therapeutic avenues to treat ageing-related diseases, such as AD.

## ROLE OF ENDOPLASMIC RETICULUM STRESS IN ALZHEIMER'S DISEASE-ASSOCIATED ENDOTHELIAL DYSFUNCTION

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Chronic activation of the unfolded protein response of the endoplasmic reticulum (UPR<sup>ER</sup>) under stress conditions has been associated with dysfunction and loss of several cell types, and it is considered an early pathogenic event in Alzheimer's disease (AD). Therefore, the prolonged UPR<sup>ER</sup> activation may be implicated in the AD-associated degeneration of the cerebrovascular unit. However, the mechanisms underlying endoplasmic reticulum (ER) stress-induced brain endothelial cells (ECs) demise are not completely understood. To fill this gap we hypothesized that: (1) chronic UPR<sup>ER</sup> activation leads to brain ECs dysfunction and death through changes in amyloid precursor protein (APP) processing and amyloid beta (A $\beta$ ) generation and modification of tau through phosphorylation. (2) Alterations of tau phosphorylation status, via inhibition of protein phosphatase 2A (PP2A), culminate in the rearrangement of endothelial cytoskeleton, compromised ER-mitochondria communication, impaired Ca<sup>2+</sup> and redox homeostasis and mitochondrial dynamics, finally leading to the activation of apoptotic cell death. Our studies revealed that prolonged ER stress induced by the treatment of rat brain ECs (RBE4) with two well-known ER stress inducers, thapsigargin and brefeldin A, led to intracellular APP accumulation, which co-localizes with the ER marker glucose regulated protein 78 (GRP78), and activated  $\beta$ -secretase leading to accumulation of intracellular A $\beta$ . These alterations occurred concomitantly with the abnormal phosphorylation of the microtubules (MTs)-associated protein tau. Taking into account that PP2A regulates tau protein phosphorylation and is inhibited in AD brains, RBE4 cells were also exposed to okadaic acid (OA), an inhibitor of PP2A. Under these conditions, RBE4 cells showed MTs destabilization, which was associated with decreased levels of acetylated tubulin and actin filaments rearrangements. Alterations of RBE4 cytoskeleton were correlated with the activation of the ER stress response and a pronounced ER-to-mitochondria Ca<sup>2+</sup> transfer, ending in mitochondrial Ca<sup>2+</sup> overload and alteration of mitochondria dynamics. In conclusion, chronic activation of ER stress-induced UPR<sup>ER</sup> and abnormal tau phosphorylation, via PP2A inhibition, form a vicious cycle that propagates ECs dysfunction. Moreover, data support the hypothesis that tau protein interacts (in)directly with actin microfilaments promoting the bundling and accumulation of F-actin, which disrupts mitochondria-ER contacts promoting cell death.

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## OXIDATIVE STRESS INVOLVING CHANGES IN NRF2 AND ER STRESS IN EARLY STAGES OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) progression has been consistently associated to oxidative stress and endoplasmic reticulum (ER) dysfunction. Using human peripheral blood mononuclear cells (PBMCs) and both brain samples and PBMCs from AD transgenic mouse model at different disease stages, we proposed to evaluate whether oxidative stress involving Nrf2 deregulation and ER stress may constitute early events in AD pathogenesis. In PBMCs isolated from individuals with mild cognitive impairment (MCI) we observed increased ROS levels, which correlated with upregulation of phosphorylated Nrf2 (p(Ser40)Nrf2), a ROS-related transcription factor. Moreover, impaired ER Ca<sup>2+</sup> homeostasis and increased ER stress markers were observed in PBMCs from MCI individuals and mild AD patients. Results obtained in the young (3 month-old) 3xTg-AD substantiated the evidence of early oxidative stress. Indeed, we observed increased p(Ser40)Nrf2 levels in PBMCs and increased nuclear Nrf2 levels in the brain cortex of 3 month-old 3xTg-AD *versus* age-matched non-transgenic male mice. Nevertheless, SOD1 protein levels were decreased both in human MCI PBMCs and 3xTg-AD mice brain cortex; the latter further correlated with reduced SOD1 mRNA levels. Increased ER stress was also detected in the brain cortex of young female and old male 3xTg-AD mice. In summary, we demonstrate oxidative stress and early Nrf2 activation in AD human and mouse models, which fails to regulate some of its targets, leading to repressed expression of antioxidant defenses (e.g. SOD-1), and extending to ER stress. Our results suggest markers of prodromal AD linked to Nrf2 dysfunction, oxidative and ER stress that may be followed in human PBMCs.

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## **BDNF INCREASES SURFACE EXPRESSION OF GLUN2B-CONTAINING NMDA RECEPTORS BY A MECHANISM INVOLVING SYNAPTIC SYNTHESIS/ACTIVATION OF PYK2**

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Synaptic plasticity events are considered the cellular correlate of learning and memory, and alterations in these mechanisms underlie several neurological and psychiatric disorders. Therefore, cognitive enhancement is considered a valid treatment strategy. In particular, overexpression of NMDA receptor subunit, GluN2B, was shown to improve synaptic plasticity and memory. However, the mechanisms of regulation of the NMDA receptor GluN2B subunit are poorly understood. In this work we investigated the molecular mechanisms involved in the regulation of synaptic GluN2B-containing NMDA receptors by the neurotrophin brain-derived neurotrophic factor. We found that BDNF increases the surface expression of GluN2B by a mechanism dependent on the proline-rich tyrosine kinase 2 (Pyk2). Pyk2 is a kinase highly expressed in central nervous system, belonging to focal adhesion kinase (FAK) family. Studies performed in cultured hippocampal neurons and in hippocampal synaptoneurosomes showed that BDNF increases synaptic synthesis of Pyk2 by a mechanism involving the RNA-binding protein, hnRNPk, resulting in an accumulation of this kinase in the synaptic compartments. Moreover, we found that the accumulation and synaptic activation of Pyk2 is required for the BDNF-induced increase in surface expression of GluN2B-containing NMDA receptors in hippocampal neurons. Pyk2 was also found to play a role in the control of GluN2B surface expression in cultured hippocampal neurons under resting conditions. We hypothesize that Pyk2 and its effects on surface expression of GluN2B subunit may contribute to the BDNF-evoked increase in the density of excitatory synapses. Taken together, our results suggest that BDNF induces synaptic activation/accumulation of Pyk2 by a mechanism involving hnRNPk and dendritic Pyk2 synthesis, resulting in an enhancement on surface levels of GluN2B-containing NMDAR. This mechanism may mediate the effects of BDNF on synaptic plasticity and may constitute a novel therapeutic target to restore the learning and memory deficits exhibited in some brain disorders. (Supported by FCT, Portugal)

## **CARBON MONOXIDE AS A METABOLIC AND BRAIN-BLOOD BARRIER MODULATOR WITH PROTECTIVE EFFECTS AFTER STROKE**

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Stroke is the main cause of brain damage in Portugal, leading to mortality and morbidity. There is presently a critical and increasing need for the development of efficient strategies against it. Ischemic strokes (corresponding to 87% of all strokes) induce cerebral damage due to oxygen and tissue energy depletion, which leads to acidosis, neuroinflammation, glutamate excitotoxicity, and generation of ROS. Astrocytes are the most metabolic active cells in the Central Nervous System (CNS) and are involved in brain structural and functional support, maintenance of normal neuronal neurotransmission and metabolism, as well as in repair after trauma. Thus, promoting astrocytic protection can be a promising therapeutic strategy.

Carbon monoxide (CO) is an endogenous gasotransmitter produced by haem-oxygenase (HO) cleavage of haem group. For the last 15 years, CO involvement in cytoprotection and anti-inflammation has been studied in several diseases and tissues. In the case of brain injury, exogenous CO prevents astrocytic and neuronal cell death by: (i) limiting mitochondrial membrane permeabilization and the release of pro-apoptotic factors into the cytosol and (ii) by stimulating mitochondrial metabolism and oxidative phosphorylation.

In this project we are investigating the putative role of CO-induced brain cytoprotection following cerebral ischemia, using cell cultures and an in vivo model of transient focal ischemia. First we performed sequencing of whole transcriptome from primary cultures of astrocytes treated with exogenous CO, as a strategy to identify the molecular effectors of CO-induced cytoprotection. Several candidate genes were validated and we are presently investigating FosB pathway activation induced by CO as well as its relevance on CO effect on cytoprotection. We are also investigating the putative neuroprotective effects of CO when administered after transient focal ischemia induced by middle cerebral artery occlusion (tMCAo).

The experimental design includes 3 doses of CORM-A1 (a CO-releasing molecule) given i.p. at 6h±12min, 32h±5h and 56h±7h after occlusion; MRI acquisitions 1day and 7 days after occlusion and evaluation of motor performance every 2 days starting 1 day after occlusion until the 7<sup>th</sup> day. The effects of CO were further evaluated by behavioural analysis. A slight motor improvement as well as an increased integrity of the blood-brain barrier was observed in animals treated with the CO releasing molecule. CO also prevented the metabolite load loss at an early stage after injury.

In conclusion, this work allows integrating data from transcriptome, behaviour, metabolism and vasculature analysis, and is expected to contribute to the understanding of the effects of CO treatment in brain ischemia.

**KEYWORDS:** Carbon monoxide, brain, ischemia, apoptosis, astrocytes, metabolism

## THE SYNAPTIC PROTEOME: A FUNCTIONAL AND QUANTITATIVE STUDY

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Local mRNA translation is an essential mechanism to control the local proteome in response to extracellular signals in axons and dendrites. This phenomenon provides extensive advantages over the transport of pre-existing proteins. Indeed, a role has been established for local mRNA translation in axonal growth, injury-induced responses and brain plasticity. Contact between a pre- and postsynaptic target is followed by several events including the recruitment and assembly of molecules that contributes for a proper synapse formation. The fibroblast growth factor 22 (FGF22) is a presynaptic organizing molecule in the CNS, regulating the formation of glutamatergic synapses. Dysregulation of FGF22 signaling during development has been proposed to increase vulnerability to neuropsychiatric disorders, including epilepsy. However, the signaling pathways activated in response to FGF22 are not clear. In this work we investigated the signaling pathways that are activated by FGF22 and identified the pool of newly synthesized proteins.

We found that FGF22 induces robust activation of the PI3K/Akt and MEK/Erk pathways in hippocampal neurons in culture. Moreover, inhibiting any of these pathways with the corresponding pharmacological inhibitors blocks FGF22-induced synaptogenic effect, and the same was observed in neurons transfected with shRNA against Akt and Erk. Also, here we report the stable isotope labeling by amino acids in cell culture (SILAC) as a novel method to directly quantify and measure protein turnover in a pure axonal preparation. To accomplish this, a SILAC-based proteomic approach was used to measure changes in the composition of synaptosomes from rat cortical neurons labeled with Lys8/Arg10 upon FGF22 stimulation. We found that FGF22-induced newly synthesized proteins compose ~3% of the synaptosomal proteome. These include a diverse set of synaptic vesicle trafficking proteins, binding and receptor associated proteins. Our data provide new insights into the FGF22 locally induced proteome at the synapse, a prerequisite for understanding the functional and molecular dynamics between synaptic contacts.

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## IDENTIFICATION AND CHARACTERIZATION OF A NOVEL MICRORNA REGULATED BY NEURONAL ACTIVITY

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Synaptic plasticity is at the basis of processes such as learning and memory. The number and strength of synapses are altered in an activity-dependent manner that relies on long-term modifications in gene expression and local protein synthesis, through mechanisms and regulators that are still largely unclear.

MicroRNAs, well known posttranscriptional regulators, have been shown to have a key role in the control of local translation in neurons since they can be rapidly modified by neuronal activity. Disruption of these mechanisms can lead deficiencies in neuronal development and synaptic plasticity. Our main goal is to unveil and characterize novel activity regulated microRNAs that are involved in regulating the expression of plasticity-relevant transcripts.

We have performed gene expression microarray analysis of rat hippocampal neurons under blockade of neuronal activity. Focusing on a limited group of altered genes with crucial roles in plasticity, we predicted microRNA target sites and performed a screening panel for these microRNAs expression levels in primary cultures of rat hippocampal neurons subjected to manipulation of neuronal activity. This screening allowed us to identify several novel activity regulated microRNAs, miR-186 in particular exhibits a dramatic activity-dependent change in its expression levels and regulates the expression of neuronal targets with a role in plasticity. Gain or loss of function studies ascertains a role for miR-186 in regulating neuronal function.

## DIPEPTIDYL PEPTIDASE IV INHIBITION PREVENTS ADIPOCYTE DIFFERENTIATION AND AMELIORATES FIBROSIS IN ADIPOSE TISSUE OF OBESE MICE

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Obesity is characterized by an excessive increase of white adipose tissue, causing local hypoxia in expanding adipocytes [1]. Rapid expansion of adipose tissue in obesity also promotes adipose tissue extracellular matrix remodeling, leading to fibrosis formation [2]. Moreover, dipeptidyl peptidase IV (DPPIV) inhibitors (gliptins) are oral antidiabetic drugs that are commercially available. DPPIV inhibitors were shown to prevent myocardial fibrosis in diabetic mice [3]. Therefore, this study aims to unravel the role of DPPIV inhibitors in fibrosis that occur in white adipose tissue in obesity. In the *in vitro* study, hypoxia was mimicked in 3T3-L1 murine pre-adipocytes, by treating the cells with CoCl<sub>2</sub> (100 μM). Fibrosis was induced by treating cells with transforming growth factor β1 (TGFβ1; 2.5 ng/mL) in the presence or absence of DPPIV inhibitors, vildagliptin (2 nM), sitagliptin (20 nM) or saxagliptin (1 nM). In the *in vivo* study, we used C57Bl/6J male mice that were fed a high fat diet (HFD) or a chow diet and treated with the DPPIV inhibitor, vildagliptin (30 mg/kg/day), for eight weeks. Our results show that, in adipocytes, the hypoxia mimetic CoCl<sub>2</sub> increases fibrosis markers: fibronectin, α smooth muscle actin and collagen VI. Moreover, the fibrosis induced by TGFβ1 increases lipolysis but decreases adipocyte differentiation. DPPIV inhibitors (vildagliptin, sitagliptin or saxagliptin) prevent TGFβ1-induced fibrosis, but also inhibit adipogenesis. In the *in vivo* study, vildagliptin improves fasting blood glucose, glucose tolerance and blood cholesterol and triglycerides in HFD-fed mice. Vildagliptin does not change body weight but decreases fat weight. Moreover, vildagliptin prevents the extracellular matrix changes that occurred in the HFD-fed mice. Taken together these results suggest, for the first time, that DPPIV inhibitors prevent adipocyte differentiation and may decrease fibrosis in adipose tissue induced by obesity.

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## SPERMATOGENIAL STEM CELL ORGANIZATION IN FELID TESTIS AS REVEALED BY DBA LECTIN

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# Sara Escada-Rebelo S and Andreia F Silva contributed equally to this work

Spermatogonial stem cells are being exploited in many species as a tool to recover fertility. Nonetheless, they may also be used to manipulate the genetic pool of the descendants. In either situations knowledge of these cells characteristics is mandatory and as so, our objective was to improve it in the domestic cat, used as animal model for endangered felid species and to some human diseases/physiological processes. For this purpose we have screened several markers, that could be used to distinguish and study the undifferentiated spermatogonia population *in situ* and *in vitro* through immunohistochemistry applied to tissue sections and whole mounts of domestic cat seminiferous tubules. Our results showed that PGP9.5 and FoxO1, although appropriate to label the cytoplasm and nucleus of gonocytes and spermatogonia in pre-pubertal animals, cannot be considered markers of undifferentiated spermatogonia in adult animals as almost all spermatogonia, namely type A and B, express these proteins. Nonetheless, the lectin of *Dolichos biflorus* Agglutinin (DBA) was able to label the cell surface and cytoplasm of a small type A spermatogonial population in the adult animals. Analysis of the number and distribution of the DBA-labeled cells showed they were present in low number and that their number did not vary with epithelium seminiferous stage. Morphometric analysis revealed that DBA-labeled cells present tropism to a peculiar area of the seminiferous tubules, namely that in direct contact with Leydig cells. Whole mounts of DBA-stained seminiferous tubules disclosed the arrangement of these cells in small clones up to 8 cells. Noteworthy, the clonal cells presented variable staining intensity suggesting the existence of asymmetric distribution of O-glycosylated proteins within each clone.

Our results strongly point toward DBA lectin as a marker of undifferentiated spermatogonia in domestic cat and illustrate the differences in SSCs development and organization present in this species.

## FUNCTIONAL GENOMICS ANALYSIS TO GATHERING EVIDENCE FOR m.7486G>A MUTATION PATHOGENICITY

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More than 20 variants, in 13 different mitochondrial transfer RNAs, are reported as a genetic cause of a Chronic Progressive External Ophthalmoplegia (CPEO) phenotype, characterized by slowly progressive paralysis of the extraocular muscles.

The novel m.7486G>A (*mt-TS1* gene) alteration was detected in a patient with CPEO diagnosis, by automated sequencing at the Biochemical Genetics Laboratory (LBG, CNC-Faculty of Medicine, Coimbra). According to *in silico* analysis using bioinformatics tools, this alteration is probably pathogenic.

In order to understand the functional impact of the novel alteration in the pathophysiology of the disease, some genetic, biochemical and functional studies were performed.

First, the sequence variation was confirmed by Next Generation Sequencing (at the Baylor College of Medicine, Houston) and by pyrosequencing at Mitochondrial NCG Diagnostic Laboratory, Newcastle. It is present in muscle, lymphocytes and fibroblasts with 50%, 4% and 10% of heteroplasmy, respectively. The mitochondrial respiratory chain activity was measured and a decreased activity in several complexes was detected, being the muscle the most affected tissue. The presence of COX-deficient fibres in this tissue allowed to perform single fibre studies, the gold standard method to prove the pathogenicity of mitochondrial transfer RNA alterations. It was also possible to confirm that the alteration segregates with COX-deficient fibres. Besides, functional studies were performed in patient's and controls' fibroblasts to evaluate metabolic changes. The results obtained by fluorimetry suggest mitochondrial membrane depolarization and increased superoxide production inside mitochondria. The mitochondrial ATP levels, the basal respiration and the maximal respiratory capacity are decreased and the glycolysis and the glycolytic capacity are increased showed by the Seahorse Bioscience analysis. Moreover, the electron microscopy revealed that patient's fibroblasts present abnormal intracellular structures.

The results suggest mitochondrial dysfunction in patient's fibroblasts and segregation of the mutation in COX-deficient muscle fibres. These facts highlight the high probability that the m.7486G>A variant is related to the pathogenicity of the disease.

## ADJUVANT NANOCARRIERS FOR HEPATITS B VACCINE

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New strategies to increase HBV vaccination, especially in developing countries, have been presented by the scientific community. Among them, the development of new adjuvants and new vaccine formulations for unusual vaccine administration routes has demonstrated great value. Nanoparticles (NPs) prepared from natural or synthetic polymers are extremely attractive vaccine adjuvants able to promote antigen delivery and presentation and in some cases hold intrinsic immunostimulatory properties. In particular, poly- $\epsilon$ -caprolactone (PCL)/chitosan NPs have a hydrophobic character which is a key characteristic for activating receptors of pathogen associated molecular patterns (PAMPs). Also, chitosan, a mucoadhesive biopolymer has been extensively described for its immunostimulatory properties.

The purpose of the present work was to prepare poly- $\epsilon$ -caprolactone (PCL)/chitosan NPs and to investigate their adjuvant effect for the recombinant hepatitis B antigen (HBsAg) and plasmid DNA (pDNA) encoding HBsAg (pRC/CMV-HBs). Particles were prepared by simple nanoprecipitation. Final vaccine formulations were achieved by simple incubation of PCL/chitosan NPs with HBsAg and/or HBsAg encoding plasmid.

PCL/chitosan NPs were positively charged in water (+ 25 mV) with a mean diameter of approximately 200 nm and presented reduced cytotoxicity for spleen cells isolated from healthy mice. Uptake studies performed in alveolar epithelium (A549) and colorectal epithelium (Caco-2) cell lines and in peripheral blood mononuclear cells (PBMC) isolated from human blood revealed PCL/chitosan NPs were efficiently internalized, an important feature for efficient vaccine delivery systems.

*In vitro* mechanistic assays testing  $\beta$ -hexosamidase release from HMC-1 cells and TNF- $\alpha$  production by PBMCs were performed under LPS-free conditions. Particles did not induce TNF- $\alpha$  secretion from PBMCs but they induced  $\beta$ -hexosaminidase release from mast cells (HMC-1) demonstrating its immunomodulatory capability.

Vaccination studies were performed in C57BL/6 mice using different strategies to evaluate: (1) PCL/chitosan NPs adjuvant ability; (2) PCL/chitosan NPs dose effect, (3) subcutaneous and intranasal administration routes; (4) the antigen dose effect. Mice serum samples, nasal and vaginal washes were analyzed for anti-HBsAg specific immunoglobulins. Mice spleen cells were cultured with or without the antigen to evaluate cytokine production. Immune response evaluation was based on enzyme-linked immunosorbent assay (ELISA). Results showed vaccination with HBsAg encoding plasmid adsorbed on PCL/chitosan NPs conducted to a negligible immune response, a result we speculate to be related to the highly negative zeta potential presented by the final formulation. Alternatively, the recombinant HBsAg protein adsorbed on the surface of NPs administered subcutaneously led to a strong humoral immune response (IgG titers), superior to the response generated by vaccination with the commercial vaccine Engerix-B®, at the same dose ( $p < 0.05$ ). Results also revealed the NPs ability to promote a specific cellular immune response based on the production of IFN- $\gamma$  and IL-17 by spleen cells, contrarily to what was observed with free HBsAg. These increased immune responses were proved to be dependent on the NPs dose, supporting its adjuvant immunostimulatory ability. Intranasal vaccination with HBsAg adsorbed PCL/chitosan NPs showed advantages since an antigen dose equivalent to the one used for the subcutaneous route (1.5  $\mu$ g antigen/mouse) was able to induce humoral immune responses.

Altogether, our results confirm the ability of PCL/chitosan NPs as a vaccine adjuvant for HBsAg. Moreover, although the extrapolation of these results to other recombinant antigens presents some limitations, the conclusions drawn here are of great importance for future applications of PCL/chitosan NPs.

## FLEXIBLE NANOFILMS COATED WITH ALIGNED PIEZOELECTRIC MICROFIBERS PRESERVE THE LONG-TERM CONTRACTILITY OF CARDIOMYOCYTES

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**Introduction:** Few examples have reported the successful use of engineered cardiac tissue for drug screening/toxicology assessment. This issue is of paramount importance since cardiac toxicity has been implicated in 28% of drug withdrawals over the last 30 years. The development of tissue engineered cardiac tissue for drug screening requires the development of scaffolds that can be easily produced, flexible, small, and preserve the long-term contractility of cardiomyocytes, ideally in the absence of complex external electrical stimulation apparatus. Here we develop a flexible scaffold relatively easy to prepare, that reproduces aspects of cardiac ECM, and can preserve the contractility of fetal rat cardiomyocytes for high-throughput drug screening applications. The scaffold is formed by a nanofilm of poly(caprolactone) (NF) coated by piezoelectric microfibers (PIEZO) composed of poly(vinylidene fluoride–trifluoroethylene) (PVDF-TrFE). When a mechanical force is applied to a piezoelectric material a shift or rotation of the constitutive dipole crystals occurs resulting in the generation of an electric charge. Therefore, PIEZO fibers may act as Purkinje cells, which in the native tissue are responsible for initiating and synchronizing cardiac beatings.

**Results and Discussion:** To evaluate whether NF+PIEZO scaffolds preserve CM contractility, the number of spontaneous synchronous beating per minute was monitored at day 1 and 12 cell post-seeding. While the average rate of beats on cells cultured in poly(styrene) and NF scaffolds maintained constant from day 1 to day 12, a significant increase in beats /minute of the cells cultured in NF+PIEZO scaffolds (from 18 to 106 beats/min) was observed. This indicates that, for at least 12 days, NF+PIEZO scaffold provided a better environment to preserve the spontaneous contractility of CMs. Alongside, cells cultured in NF+PIEZO scaffold displayed high levels of functional connexin 43 and waveform-like electrochemical signalling, which denote proper cell-cell communication and action potential conduction. When compared to tissue culture poly(styrene), the piezoelectric scaffold promoted transmembrane transients of Ca<sup>2+</sup> as seen by high concentration of intracellular Ca<sup>2+</sup> and greater expression of important transmembrane ion channels. These observations were accompanied by clear morphological changes including a 3-fold increase in CM alignment, highly organized sarcomeric structures and a higher CM surface area than the control. In addition, a main component of the contractile machinery ( $\alpha$ -cardiac actin) was up-regulated, while its fetal counterpart ( $\alpha$ -skeletal actin) dropped significantly in the piezo scaffold.

To evaluate the usefulness of our platform for drug screening, norepinephrine was applied revealing an expected rise of intracellular Ca<sup>2+</sup> concentration accompanied by an increase in beating rates. Finally, metabolic assessments demonstrated that the piezo scaffold favours a more efficient (aerobic) metabolism. This effect is negated by the presence of the cardiotoxic drug, doxorubicin, which forces a transition to a glycolytic (anaerobic) metabolism and induces high cell death. In overall, these results demonstrate the positive effect of the piezo scaffold on cardiac cell function. Furthermore, it reveals the sensitivity of the engineered tissue towards toxic insults, thus providing proof of the value of this platform for screening drug candidates.

## **RLR1 AND RLR2, TWO NOVEL *ARABIDOPSIS THALIANA* ATYPICAL ASPARTIC PROTEASES INVOLVED IN PRIMARY ROOT DEVELOPMENT AND LATERAL ROOT FORMATION**

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Aspartic proteases (APs) represent the second largest class of plant proteases after serine proteases. Members of the pepsin-like family are widely distributed in plants, e.g. 70 APs homologues are found in *Arabidopsis thaliana* and 166 in *Oryza sativa*, the vast majority of them having atypical characteristics and properties (atypical APs). This contrasts strikingly with the considerably fewer number of APs encoded in the mammalian genomes (e.g. *Homo sapiens* has only 17 APs). The overrepresentation of APs in plants suggests potentially important and diverse roles for these proteins. Although the AP class of proteases has been much less studied than other classes of proteases, some functions are starting to be uncovered, with proposed roles in highly regulated processes like resistance to biotic/abiotic stresses, PCD, plastid homeostasis, and reproduction, which is consistent with functional specialization of plant APs and tight activity regulation. In this work, two *Arabidopsis* AP genes were characterized. Phenotypic analysis of T-DNA insertion mutants for each gene revealed significant reductions in primary root length and in lateral root number. Moreover, these phenotypes were evaluated under nutrient limitation, with results suggesting that these genes may be involved in two different regulatory mechanisms of lateral root formation. Therefore, these genes were designated RLR1 and RLR2 (Regulator of Lateral Root). Moreover RLR1 and RLR2 overexpression mutants also show de-regulation of lateral root formation and primary root growth under different growth conditions further strengthening the importance of these atypical APs in both mechanisms. RLR1 gene product was produced using the innovative plant-based expression platform magniCON® in *Nicotiana benthamiana* leaves and biochemically characterized. RLR1 was shown to be glycosylated, active at acidic pHs, not completely inhibited by pepstatin A, and with a distinct specificity pattern determined by Proteomic Identification of Protease Cleavage Site (PICS). Redox agents have also a significant inhibitory effect on RLR1 activity suggesting that this protein might be involved in redox sensing mechanisms. These results clearly demonstrate that RLR1 is an AP with distinct and atypical biochemical properties. Identification of putative substrates of RLR1 and RLR2 using iTRAQ and Terminal Amine Isotopic Labeling of Substrates (TAILS) analysis is currently ongoing and will give the first insights into the molecular pathways where these proteins are operating.

Our results unveil a new role for aspartic proteases in the regulation and adaptation of root development in *Arabidopsis* under normal growth conditions as well as under abiotic stresses.

# **Abstracts for Poster Presentations**



## NEW BIOMARKERS FOR OXIDATIVE STRESS: A MASS SPECTROMETRY COMPREHENSIVE CHARACTERIZATION OF CELLULAR SECRETOME

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### Introduction and Objectives

The proteins secreted by cells play important roles in cellular communication and in the regulation of many physiological processes, being therefore good predictors of the cellular physiological state. As a result, the secretome is an important source of potential biomarkers and a good target for therapeutics. Oxidative stress is perhaps the most common factor involved in a large variety of disorders, however oxidative stress (de)regulation may occurs through different mechanisms, and thus leading to different cellular responses, which can be reflected in the secretome. These alterations can be associated with: the secretion of new factors; differential secretion of particular factors; and different oxidative states of the secreted proteins. Therefore, in this work it is presented a comprehensive method to evaluate the secretome changes and to integrate protein levels with their oxidative state changes.

### Methods

A cell model was treated with hydrogen peroxide, and the newly generated secretome was spiked with the appropriate internal standards, and analyzed by SWATH-MS.

The entire secretome was used to count for differences in the total amount of secreted protein. Finally, differential alkylation was used to evaluate the oxidative state of the proteins.

### Results and Discussion

A large number of proteins and small molecules were quantified between control and oxidative stress condition, which allows to obtain a clear distinction between the two conditions. More importantly, the use of exogenously added internal standards, generated from our group, allowed better data normalization. Considering the toxic conditions used, it is important to determine if the protein content change is due to membrane disruption. Therefore, a careful analysis on endogenous proteins revealed a panel which can be used as cellular integrity standards. Finally, a SWATH-MS approach was developed to quantify the cysteine redox dynamics in the secretome allowing now to monitor hundreds of proteins in a single analysis.

### Conclusion:

An integrative approach was introduced, and successfully applied in the identification of oxidative stress biomarkers in cellular secretome.

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## NEUROPEPTIDE Y RESCUES THE SENESCENT PHENOTYPE OF HUMAN HUTCHINSON-GILFORD PROGERIA SYNDROME CELLS

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Hutchinson-Gilford progeria syndrome (HGPS), a lethal genetic disorder, is characterized by premature aging. HGPS is most commonly caused by a *de novo* point mutation (C608G) within the lamin A/C gene (LMNA), producing an abnormal lamin A protein termed progerin. Accumulation of progerin causes nuclear abnormalities and cell cycle arrest, ultimately leading to cellular senescence. It has been shown that rapamycin, by stimulating autophagy, promotes progerin clearance and has beneficial effects on HGPS cell models. Since rapamycin has well-known adverse effects, the identification of safer stimulators of autophagy, with other beneficial effects, for chronic treatment of HGPS patients is of utmost importance. Neuropeptide Y (NPY) and NPY receptors (NPY Y<sub>1</sub>-Y<sub>5</sub>) are expressed throughout the body and have been shown to regulate different functions with relevance for HGPS pathology (i.e, angiogenesis, bone remodeling protection, myocardial repair, stem cells proliferation). Moreover, we have recently showed that NPY, similarly to rapamycin, increases autophagy (Aveleira, Botelho *et al.*, 2015) in the hypothalamus, a brain area recently identified as a central regulator of peripheral aging (Zhang *et al.*, 2013). In addition, we also observed that NPY mediates caloric restriction-induced autophagy (Aveleira, Botelho *et al.*, 2015). These results are in accordance with some previous studies suggesting that NPY may play a role as a lifespan and as an aging regulator. In fact, Chiba *et al.*, (2014) showed that caloric restriction, one of the most robust anti-aging interventions, does not increase lifespan in NPY knockout mice. Moreover, other studies suggest that NPY might act as a caloric restriction mimetic (Minor *et al.*, 2008), supporting NPY's anti-aging role. Taking all this into account, the aim of the present study was to investigate if NPY could be a relevant strategy to delay the premature aging phenotype of HGPS.

Primary cultures of dermal fibroblasts derived from HGPS patients were used as cell model. Cells were exposed in a time-dependent manner to NPY and its effects on several cellular aging hallmarks were evaluated: 1) progerin accumulation; 2) autophagy impairment; 3) nuclear abnormalities; 4) DNA damage; 5) decreased cell proliferation; and 6) cellular senescence.

In HGPS cells, NPY induced a 70% decrease in progerin protein levels, concomitant with an increase of autophagic flux. In addition, NPY decreased the number of dysmorphic nuclei, a hallmark of HGPS cells, and decreased γH2AX foci, a marker of DNA damage. We also observed that NPY increased HGPS cells proliferative capacity, as determined by an increase of 60% in the number of Ki-67-positive cells and a decrease of p53 and its downstream effector p21, well known cell cycle inhibitors. Moreover, fewer senescence associated-β-galactosidase-positive cells were observed in NPY-treated cells, indicating that NPY slowed down the progression of cellular senescence.

Altogether, these results show that NPY rescues several hallmarks of cellular aging of HGPS cells, suggesting that NPY can be considered a promising strategy to delay or block the premature aging of HGPS. Given the plethora of NPY actions, related to aging, this study also suggest that NPY could be a potential therapeutic strategy not only for HGPS but also for normal aging or age-related diseases.

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## ACTION OF TOLBUTAMIDE ON TEA-EVOKED ZINC CHANGES FROM HIPPOCAMPAL CA3 PYRAMIDAL NEURONS

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The hippocampal mossy fibers contain a large amount of chelatable zinc in the glutamatergic vesicles, which is co-released with glutamate during synaptic transmission. Intense chemical stimulation, caused by the application of tetraethylammonium (TEA), induces long-term potentiation (LTP) at the mossy fiber synapses of CA3 area. The aim of this work was to determine the effect of TEA and the role of ATP-dependent potassium channels (KATP) in synaptic zinc activity, using tolbutamide, an inhibitor of these channels.

Optical zinc signals were detected at the mossy fiber synapses in area CA3 of hippocampal slices (400  $\mu\text{m}$ ), from pregnant (16-18 days of gestation) female Wistar rats (10-13 weeks old) using the fluorescent zinc indicator Newport Green ( $K_D = 1 \mu\text{M}$ ). The application of TEA (25 mM) caused a depression of the zinc signals ( $8 \pm 1 \%$ ,  $n = 6$ ), that might, at least in part, be due to the activation of presynaptic KATP channels by released zinc. This depression was reduced ( $4 \pm 1 \%$ ,  $n = 3$ ) in the presence of TEA (25 mM) plus tolbutamide (250  $\mu\text{M}$ ). Following washout of both media the zinc signals increased reaching a stable level above baseline. These results suggest that the KATP channels are involved in the TEA-evoked zinc depression. This inhibition is followed by a potentiation upon returning to the normal medium, that likely accompanies the expression of TEA-induced mossy fiber LTP.

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## STARGAZIN, A NEW CANDIDATE FOR SCHIZOPHRENIA - IDENTIFICATION OF NEW VARIANTS AND TARGETED THERAPIES

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Schizophrenia is a devastating disorder that affects about 1% of the worldwide population. This multifactorial disease, with a strong genetic component, is characterized by delusions, hallucinations and confusion thoughts. Furthermore schizophrenia onset and stress are inextricably related. It is well accepted that schizophrenia patients are vulnerable to changes in the environment and their ability to adapt to those changes is disrupted. Recently, synaptic networks components have been strongly implicated in this disorder as several *de novo* mutations have been found in these patients.

Stargazin is an auxiliary subunit for AMPAR and it is required for AMPAR trafficking to the surface, through its interaction with PSD95. Our lab recently described an important role for stargazin in experience-dependent plasticity and scaling up of AMPAR upon blockade of activity. Interestingly, SNPs that confer susceptibility to schizophrenia were found in CACNG2 gene, which encodes for stargazin and is located in a chromosome region strongly implicated in the disorder, 22q. Using whole-genome sequence analysis, we identified a new CACNG2 variant, Stg<sup>SCZ</sup>, which, along with Stg<sup>ID</sup>, previously identified in intellectual disability, failed to deliver AMPAR to synapses. Furthermore, Stg<sup>ID</sup> was not able to respond to homeostatic plasticity-triggering stimuli. On the other hand, Stg<sup>SCZ</sup> increased the number and decreased the length of primary dendrites, as well as the number of inhibitory synapses.

Tianeptine is a commercial memory enhancer and powerful antidepressant, recently described to increase AMPAR trapping at synapses, by increasing stargazin phosphorylation. Tianeptine treatment rescued AMPAR levels in neurons expressing STG<sup>SCZ</sup>. In contrast, STG<sup>ID</sup> was unable to respond to tianeptine. We are currently generating stargazin *knock-in* mice for these mutations and we aim to explore if tianeptine can rescue circuit dysfunction caused by STG<sup>SCZ</sup> and STG<sup>ID</sup>.

## PRODUCTION AND *IN VITRO* CHARACTERIZATION OF *AB-INITIO* DESIGNED CHANNELRHODOPSIN-2 MUTANTS

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Optogenetics confers the ability to hyperpolarize or depolarize specific populations of neurons using light. Channelrhodopsin-2 (ChR2) is a light-gated cation channel derived from the microalgae *Chlamydomonas reinhardtii* that has been used as a main tool in optogenetics. The optogenetic toolbox is under continuous update with contributions from protein engineering strategies such as site-directed mutagenesis and chimeric constructs. However, some aspects of the wild-type form of ChR2 require further attention. These include the optimization of its action spectra, channel kinetics, expression levels, inactivation time, conductance and absorption peak sharpness. In terms of spectral properties, only a few variants of the protein have been successfully generated and fully characterized. ChR2 is optimally excited by blue light (470nm), which limits its use in high light-scattering biologic material such as the brain. Therefore, adjusting the ChR2 spectra towards red-shifted activation and sharpening the absorption peak are two of the most sought after properties.

In this project, we performed *ab-initio* design to propose four new ChR2 variants with tuned absorption using site-directed mutagenesis on key residues in the chromophore region. The mutations were selected with the application of Time Dependent – Density Functional Theory (TDDFT) to predict the absorption spectra of selected mutants. We also expressed and purified wild type ChR2 and four novel variants using the eukaryotic *Pichia pastoris* heterologous expression system. These new variants exhibit concordant shifts when compared to TDDFT predictions.

We are also performing additional protein characterization and assessing membrane trafficking in HEK293 cells for all new variants. Our preliminary results validate the TDDFT predictions and reveal that biophysical simulation may be used in the intelligent design of novel ChR2 variants.

**Keywords:** Optogenetics; channelrhodopsin-2; red-shift; blue-shift; TDDFT; *Pichia pastoris*

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## ALTERED MITOCHONDRIAL DYNAMICS FOLLOWING SIRT3 OVEREXPRESSION IN A CELLULAR MODEL OF HUNTINGTON'S DISEASE

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Mitochondrial dynamics is impaired in several neurodegenerative disorders, including Huntington's disease (HD). Sirtuins, NAD<sup>+</sup>-dependent lysine deacetylases, have emerged as important cellular targets that can interfere with mitochondrial biogenesis, fission/fusion, motility and mitophagy. Among them, sirtuin 3 (SIRT3) is of particular relevance, being the main deacetylase located in mitochondria. Here we evaluated the influence of SIRT3 on mitochondrial dynamics using striatal cells derived from HD knock-in mice (*STHdh*<sup>Q111/Q111</sup>) versus wild-type cells (*STHdh*<sup>Q7/Q7</sup>).

Untransfected HD cells displayed increased mitochondrial fragmentation. Concordantly, decreased levels of mitochondrial fusion proteins (Mfn2, OPA1) and increased levels of fission-related Fis1 were observed in *STHdh*<sup>Q111/Q111</sup> cells. Drp1 (also involved in mitochondrial fission) was preferentially accumulated in the mitochondrial fraction of HD cells. Increased LC3-II/I ratio, which evaluates autophagosome formation, was observed in *STHdh*<sup>Q111/Q111</sup> cells. Moreover, the autophagy adaptor p62 was found to be decreased in mutant cells, suggesting proceeding of macroautophagy. Parkin and PINK1, two markers of mitophagy, were also reduced in untransfected HD cells. No significant changes were detected in phosphorylated Parkin (required for its enzymatic activation and mitochondrial translocation). These data suggest that PINK1/Parkin-dependent mitophagy may be impaired in HD striatal cells, although it cannot be fully excluded that decreased levels of both Parkin and PINK1 might be related with increased degradation. The unbalance between mitochondrial fission and fusion observed in mutant cells was reduced after SIRT3 overexpression (OE), with decreased protein levels of Fis1 and Drp1 accumulation in mitochondria in *STHdh*<sup>Q111/Q111</sup> cells. Concordantly, an increased number of mutant cells presenting tubular mitochondria was observed after SIRT3OE. An additional significant increase in LC3-II/I ratio was observed in *STHdh*<sup>Q111/Q111</sup> cells.

-SIRT3 cells, indicative of macroautophagy activation. Data suggest that increased SIRT3 levels may restore mitochondrial morphology in mutant cells by reducing mitochondrial fission, with additional activation of macroautophagy.

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## CHRONIC HYPERGLYCEMIA IMPAIRS HIPPOCAMPAL NEWLY-GENERATED NEURONS MATURATION AND EXACERBATES MEMORY LOSS IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the leading cause of dementia in Western countries and the most prevalent neurodegenerative disease. Metabolic alterations, like in glucose metabolism, are believed to occur early in the AD onset, contributing to the development of the disease. Prolonged insults caused by chronic hyperglycemia may affect normal brain function and the capacity to cope with insults. This may lead to reduced brain cognitive reserve and capacity to compensate age-related neuronal loss, which can be related to a sustained decrease in adult hippocampal neurogenesis, early before the appearance of AD symptoms. In order to evaluate whether hyperglycemia aggravates the alterations of hippocampal adult neurogenesis in AD and further impair memory, triple transgenic AD (3xTg-AD) male mice were treated with an elevated dose of sucrose for 6 months. Upon induction of hyperglycemia, the spatial memory of 3xTg-AD mice was significantly compromised when compared to untreated age-matched 3xTg-AD mice, suggesting that hyperglycemia enhances AD-like phenotype. This exacerbation of memory loss might be related to changes in adult neurogenesis, namely the observed decrease in the total number of newly-generated neurons, reduction of dendrite complexity and number of post-synaptic (post-synaptic density 95 (PSD95)-positive) puncta in the dendritic branches of immature neurons in the dentate gyrus (DG) outer molecular layer (OML). In untreated 3xTg-AD mice, newly-generated neurons presented increased dendrite arborization and increased number of PSD95-positive puncta in the DG OML, which could compensate the decreased number of immature neurons and play an important role in the memory performance of these mice. Interestingly, hyperglycemia alone altered the morphology of the dendritic tree of immature neurons in non transgenic mice, similar to that observed in non-hyperglycemic 3xTg-AD mice, suggesting that hyperglycemia affects adult neurogenesis and may predispose to the development of AD-related memory decline. These data suggest that hyperglycemia enhances AD pathology, contributing for the impairment in neurogenesis, defective learning and memory loss.

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## **SOCIAL SUBMISSIVE BEHAVIOUR IN RANKED HIERARCHIES IS TRIGGERED BY EARLY LIFE ADVERSITY AND STRESS**

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Animals in social groups display dominant-submissive relationships that culminate in the formation of a social hierarchy. This stratification is critical for group dynamics and group fitness, as it reduces the number of intra-group conflicts through the formation of a “pecking order”. Higher-ranking, dominant individuals have priority access to food, nesting places and mate selection. In humans, it has also been shown that the perceived socioeconomic status very strongly correlates with risk for respiratory, cardiovascular, inflammatory, and psychiatric illness. Still, while genetic elements, environmental factors and prior experience all play a role in establishing social dominance, the discrete elements that predict the future rank of an individual remain conspicuously unexplored.

Early life deprivation and adversity (EDA) in rodents is used to model unfavourable rearing conditions and is known to lead to changes in anxiety-like and depressive-like behaviors. We used this paradigm to explore the role of early life environment in social hierarchy and discovered that EDA-mice display a submissive phenotype in adulthood.

These animals rank lower when compared to controls, and are more easily defeated in dominance tests. To help identify the underlying molecular targets linked to this phenotype, we performed a RNA-sequencing and discovered 180 genes with altered expression levels in the prefrontal cortex of EDA mice. From these, 20 genes seemingly correlate to the dominance index of the animals. Our on-going work centres on exploring early life adversity in its role in shaping dominance behaviour in adulthood and the underlying molecular, cellular and neuronal correlates of social hierarchy.

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## MODULATION OF INSULIN SENSITIVITY BY HYPOTHALAMIC SIRTUIN 2

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Obesity and type 2 diabetes constitute major public health problems worldwide. A central feature of these metabolic disorders is peripheral insulin resistance. Interestingly, recent studies suggest that insulin resistance also occurs in brain, in particular the hypothalamus [1, 2]. It has been proposed that improving defective hypothalamic insulin signaling and neuroinflammation may restore insulin sensitivity in peripheral organs [3]. The sirtuin family (SIRT1-7) of NAD<sup>+</sup>-dependent protein deacylases have emerged as important regulators for a variety of cellular processes, including energy metabolism, stress response and possibly aging. Data now emerging indicate that SIRT2 may also play a key role in metabolic regulation and neuroinflammation [4]. We hypothesized that hypothalamic SIRT2 may play a role in modulating insulin sensitivity. Here we show that SIRT2 is expressed in neurons of the major hypothalamic nuclei in mice. Moreover, SIRT2 levels in the ventromedial hypothalamus (VMH) are regulated by nutritional availability since its levels are markedly reduced in mice fed a high-fat diet. We also demonstrate that direct administration of palmitate, a saturated free-fatty acid, into the VMH results in reduced SIRT2 expression. Moreover, exposure of cultured hypothalamic neurons to palmitate induces insulin resistance and concomitantly downregulates SIRT2 expression. Importantly, serum starvation improves insulin sensitivity in normal conditions, and prevents the effect of palmitate on insulin resistance and SIRT2 downregulation. This study underscores the potential of SIRT2 as a future target in the prevention and/or treatment of insulin resistant states.

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## SYNAPTIC AND CELLULAR CHARACTERIZATION OF GPRASP2 AS A NOVEL CANDIDATE SUSCEPTIBILITY GENE FOR AUTISM

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Autism and autism spectrum disorders (ASDs) are neurodevelopmental disorders diagnosed based on a triad of criteria: deficits in communication, impaired social interactions, and repetitive or restricted interests and behaviours. Recent genetic and genomic studies have identified a large number of candidate genes for ASDs, many encoding synaptic proteins, that converge on ionotropic and metabotropic glutamate signaling. Presently, several lines of evidence suggest that metabotropic glutamate receptors (mGluRs) play an important role in ASD pathophysiology. Nevertheless, research work centering on the proteins that directly regulate the trafficking and surface availability of mGluRs has not been widely explored.

The G Protein-Coupled Receptor Associated Sorting Protein (GPRASP) family regulates the trafficking of diverse classes of G-protein coupled receptors, including the mGluR1 and mGluR5 receptors. These GPRASP proteins are involved in endocytic sorting of G-coupled protein receptors towards lysosomal degradation. From these, GPRASP2 in particular has recently been proposed as a susceptibility gene for autism. Here we describe our progress in characterizing GPRASP2, its location in subcellular compartments, expression in different brain regions and across development. We find that GPRASP2 is enriched in glutamatergic synapses where it strongly colocalizes with PSD-95, VGLUT1 and also with mGluR1/5 receptors. Testing the functional consequences of changing levels of GPRASP2 expression, our data support a role for this protein in the regulation of neuron complexity, spine density and spine maturation. We are also investigating the crosstalk between mGluR-GPRASP2 signaling and using mouse molecular genetics to understand the circuit specific deficiencies arising from mutations in this gene.

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## REDOX DEREGLATION IN HUNTINGTON'S DISEASE YAC128 TRANSGENIC MOUSE MODEL IS UNRELATED TO MITOCHONDRIAL DYSFUNCTION

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Huntington's disease (HD) is an inherited dominant autosomal neurodegenerative disorder characterized by neuronal loss in particular brain regions, notably the striatum and the cortex. HD is associated with a dynamic mutation, an expansion in CAG repeats, located in the exon 1 of the *HTT* gene, associated with progressive motor disability, cognitive impairment and psychiatric symptoms. Mutant huntingtin (mHTT) expression has been associated with several pathological mechanisms, including oxidative stress and mitochondrial dysfunction. Nevertheless, the precise role of these mechanisms in HD is still not entirely understood. To address this issue, we isolated brain cortical synaptosomes and striatal and cortical mitochondria from brains of pre-symptomatic (3 months) and symptomatic (9 and 12 months) YAC128 HD transgenic (expressing full-length human mHTT) and age-matched wild-type (WT) mice. Brain mitochondria from YAC128 pre-symptomatic mice exhibited higher basal levels of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), both in the cortex and striatum, when compared to WT mitochondria. Moreover, pre-symptomatic YAC128 striatal mitochondria exhibited higher basal respiration, increased maximal respiration upon FCCP stimulation and higher levels of complex II activity in real time oxygen consumption rate experiments performed on the Seahorse XF24 analyzer. No differences were found in H<sub>2</sub>O<sub>2</sub> basal levels between WT and YAC128 cortical and striatal mitochondria at 9 and 12 months of age. Nevertheless, cortical mitochondria from 9 month-old YAC128 mice exhibited increased production of H<sub>2</sub>O<sub>2</sub> upon exposure to antimycin A (AA). At the same age, YAC128 cortical mitochondria showed reduced oxygen consumption in comparison to WT mitochondria. No differences in mitochondrial Ca<sup>2+</sup>-buffering capacity or mitochondrial membrane potential were found in YAC128 *versus* WT mice cortical mitochondria at any age tested. Cortical synaptosomes from YAC128 and WT mice with 3 months of age exhibited similar levels of H<sub>2</sub>O<sub>2</sub>, both at basal and after AA or H<sub>2</sub>O<sub>2</sub> exposure. Enhanced production of H<sub>2</sub>O<sub>2</sub> was also observed in YAC128 synaptosomes at 9 and 12 months of age, which could not be associated with differences in oxygen consumption, intrasynaptosomal Ca<sup>2+</sup> levels or altered mitochondrial membrane potential. Data suggest an inverse relation in H<sub>2</sub>O<sub>2</sub> production between brain mitochondria and synaptosomes in YAC128 mice throughout age. Indeed, higher levels of H<sub>2</sub>O<sub>2</sub> were associated with pre-symptomatic mitochondria, while in synaptosomes, H<sub>2</sub>O<sub>2</sub> production increased with age. This apparent discrepancy between mitochondria and synaptosomes may be derived from a progressive decrease of cytoplasmic antioxidant defenses during disease progression.

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## UNRAVELLING THE ROLE OF NPY ON THE CENTRAL CIRCADIAN CLOCK

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Circadian rhythms are 24h biological oscillations that virtually regulate all fundamental biological processes<sup>1</sup>. These daily rhythms are controlled by a hierarchy of biological clocks in which the suprachiasmatic nucleus (SCN), at the anterior hypothalamus, is the central regulator. The SCN is responsible for synchronizing self-sustained and independent peripheral clocks, located on almost all peripheral tissues<sup>2</sup>. At the molecular level, transcriptional and translational feedback loops of clock genes (Per1-2, Cry, BMAL1 and CLOCK) are in the basis of the clock mechanisms, although peripheral clocks rhythms may be delayed by several hours in relation to the SCN rhythm<sup>3</sup>.

The neuropeptide Y (NPY), one of the most abundant peptides in the hypothalamus, has been implicated in a vast number of physiological processes being associated with several human diseases such as obesity<sup>4</sup>. On the other hand, NPY is also an important entrainer of the SCN, being reported to advance the circadian phase<sup>5</sup>. However, its role in the circadian rhythm clock genes modulation is not clear. The aim of the present study is to investigate the role of NPY on clock genes modulation in the hypothalamus.

Immortalized hypothalamic mouse neurons (N42) were treated with NPY (100 nM) for 24 hours, at different circadian timepoints (CT; CT0h, CT4h, CT8h, CT12h, CT16h), and clock genes mRNA levels were evaluated by qRT-PCR.

The results show that NPY induces clock genes expression alterations, in a circadian time dependent manner. At CT0, we observed a highly significant increase of Per1 and a decrease of Cry genes expression. After 12 hours (CT12), NPY caused a reduction of CLOCK gene expression. And, 4 hours later (CT16), NPY induced an increase of Per1 and Per2 gene expression.

In conclusion, these preliminary results suggest that NPY modulates the central circadian clock dependent upon the circadian time.

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**Keywords:** Circadian rhythm; Hypothalamus; Clock genes; NPY

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## MITOCHONDRIAL DYSFUNCTION AND METABOLIC IMPAIRMENT IN INDUCED PLURIPOTENT AND NEURAL STEM CELLS DERIVED FROM HUNTINGTON'S DISEASE PATIENTS

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Huntington's disease (HD) is an autosomal dominant disease caused by an expansion of CAG repeats in the *HTT* gene. Several pathological mechanisms have been proposed for neurodegeneration, including mitochondrial dysfunction and oxidative stress. Cumulating evidence suggests mitochondria as an additional pathological factor in HD. An attractive model to study disease mechanisms are HD patient-derived induced pluripotent stem cells (HD-iPSC). In this study we show that HD-iPSC retaining 72 CAG repeats have hyperpolarized mitochondria due to ATP synthase reversal, which exhibit increased mitochondrial calcium accumulation, when compared to control iPSC (C-iPSC). HD-iPSC also have lower basal respiration, decreased activity of complex III and thus are highly dependent on glycolysis. Upon differentiation into neural stem cells (NSC), oxidative phosphorylation declines in both control and HD cell lines, with HD-NSC exhibiting higher impairment in O<sub>2</sub> consumption. Therefore, HD-NSC largely rely on glycolysis to generate ATP. Moreover, ATP levels are unaltered when cells are incubated with pyruvate under conditions of decreased glycolytic flux. Concordantly, we show that HD-iPSC and HD-NSC exhibit increased phosphorylation of pyruvate dehydrogenase (PDH) E1 $\alpha$  subunit at Ser232, 293 and 300, reflecting reduced PDH activity. In addition, increased levels of PDH kinase 1 are found in HD-NSC. Enhanced levels of mitochondrial superoxide anion and hydrogen peroxide are also observed in HD-iPSC *versus* C-iPSC. Moreover, HD-iPSC produce high levels of reactive oxygen species (ROS) following exposure to hydrogen peroxide, indicating increased susceptibility to oxidative stress. Nevertheless, no changes are observed in the activity of superoxide dismutase (SOD) 1 or 2 in HD-iPSC *versus* C-iPSC, although SOD1 activity increase significantly in HD cells following differentiation. Taken together, HD-iPSC resort less to oxidative phosphorylation to produce ATP, apparently due to reduced activity of PDH and mitochondrial complex III, and rely more on glycolysis, thus possessing lower ATP/ADP levels and producing more ROS. NSC maintain the same metabolic profile as iPSC, resorting more to glycolytic flux to obtain ATP, as the mitochondrial respiration is significantly decreased. Our study suggests that mitochondrial dysfunction occurs in early stages of HD cell differentiation.

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## ALTERED OXIDATIVE STRESS-RELATED PROTEINS IN HUNTINGTON'S DISEASE YAC128 MOUSE MODEL

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Oxidative stress is widely associated with neurodegenerative disorders, particularly Huntington's disease (HD). HD is an autosomal dominant neurodegenerative disorder caused by a CAG expansion in the *HTT* gene, leading to the expression of mutant huntingtin (mHTT) protein with a polyglutamine stretch at the N-terminal. HD is characterized by psychiatric, motor and cognitive symptoms, strongly affecting the striatum and the cerebral cortex. mHTT interferes with axonal transport, transcription regulation, synaptic and mitochondrial function, leading to mitochondrial calcium handling defects and oxidative stress, responsible for brain atrophy and neuronal degeneration, especially the loss of striatal medium-sized projection spiny neurons (MSNs). In this work, we studied several modifications in protein levels associated with oxidative stress in the symptomatic stages of YAC128 transgenic mice, an HD mouse model expressing human full-length mHTT with 128 glutamines. To accomplish this, we used extracts from subcellular fractionation of cortex, striatum and non-affected cerebellum of 9- and 12-month YAC128 *versus* age-matched wild-type (WT) mice. A significant decrease in antioxidant levels of peroxisomal catalase and mitochondrial superoxide dismutase (SOD)2 or Mn-SOD was observed in the cortex of 12 month-old YAC128 mice. The acetylated form at K68 of SOD2, related with diminished activity, was also found to be decreased at this age in mutant mice. Interestingly, protein levels of nuclear Nrf2, a transcription factor involved in antioxidant response, and its phosphorylated form at Ser40 (responsible for its translocation to the nucleus) were reduced in cytosolic extracts of YAC128 cortices of the same age. We also observed a decrease in phosphorylated Drp1, but unchanged levels of Mitofusin and OPA-1 in brain mitochondrial fractions; an increase in TOM-20 was also observed in the same samples. Conversely, no differences in these proteins were found in cortical or striatal extracts from 9 month-old YAC128 and WT mice. Moreover, the levels of these proteins remained unchanged in YAC128 and WT cerebelli at both ages. Data suggest that important defense mechanisms against oxidative stress are diminished in the aged brain of HD YAC128 mice.

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## MOLECULAR DYNAMIC SIMULATION OF HER2-ANTIBODIES COMPLEXES

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Human Epidermal Growth Factor Receptor 2 (HER2) is, among the EGFR family, the most relevant from a biological perspective. It remains overexpressed on tumor cells, which is associated with tumor aggressiveness and an increased probability for recurrent disease. This overexpressing occurrence develops resistance to the well-studied anti-HER2 monoclonal antibody, Herceptin®, or specific tyrosine kinase inhibitors. Thus, it is urgent to develop new approaches on anti-HER2 therapies.

In order to attain a deeper understanding of the interaction of specific anti-HER2 antibodies and HER2, computational methods modelling and Molecular Dynamic (MD) simulations were employed. The dynamic behavior of HER2 receptor in complex with three antibodies (F0178, A21 and scFv from Trastuzumab) was investigated by two replicas of 0.5  $\mu$ s MD simulations for each system as well as for the individual ones. A variety of structural characteristics ranging from pairwise interactions formation to covariance analyses are being employed. Our aim is to understand how to modify and control the formation of these macromolecular systems.

## EXCESSIVE SIRT3 ACTIVATION IS DELETERIOUS IN A HUNTINGTON'S DISEASE CELL MODEL

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SIRT3 is a stress responsive NAD<sup>+</sup>-dependent deacetylase recently shown to play a role in protecting cells under stress conditions that often occur in neurodegenerative diseases, such as in Huntington's disease (HD). This is an autosomal dominant neurodegenerative disorder clinically characterized by progressive cognitive impairment and chorea-like involuntary movements. HD is caused by an expanded polyglutamine tract in huntingtin (HTT) protein and, although the mechanisms by which neurons die are uncertain, oxidative stress and mitochondrial dysfunction have been implicated. Therefore, we speculate that targeting SIRT3 may influence HD neurodegeneration. We showed that *STHdh*<sup>Q111/Q111</sup> striatal cells, expressing mutant HTT with 111 glutamines, and human lymphoblasts derived from HD affected patients exhibited a significant increase in both SIRT3 protein and mRNA levels, along with increased SIRT3 enzymatic activity, in comparison with *STHdh*<sup>Q7/Q7</sup> wild-type cells or unaffected control lymphoblasts. Additionally, symptomatic YAC128 HD transgenic mice exhibited increased deacetylation of superoxide dismutase 2 (SOD2), a pre-recognized mitochondrial SIRT3 target, although no changes in neuronal SIRT3 protein levels were observed in this model. Remarkably, overexpression (OE) of SIRT3 in striatal cells decreased lysine acetylation and cell viability, which might be triggered by increased levels of reactive oxygen species (ROS), namely mitochondrial superoxide anion and hydrogen peroxide. Moreover, *STHdh*<sup>Q111/Q111</sup> cells displayed a significant decreased in basal respiration, compared to wild-type cells; however, SIRT3OE had no effect in this parameter. Contrariwise, the maximal respiration and the spare respiratory capacity, achieved after the stimuli with FCCP plus oligomycin to completely depolarize the organelle and prevent mitochondrial ATP hydrolysis, were significantly reduced after SIRT3OE, compared to GFP transfected control, in both wild-type and HD striatal cells. Additionally, both *STHdh*<sup>Q7/Q7</sup> and *STHdh*<sup>Q111/Q111</sup>-SIRT3 cells exhibited decreased mitochondrial membrane potential and, in the case of SIRT3-transfected wild-type cells, increased mitochondrial calcium levels. Therefore, we can speculate that exacerbated SIRT3 activity exerted negative effects in cells expressing full-length mutant HTT, which may result from higher production of ROS.

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## ANALYSIS OF CITALOPRAM IN PLASMA AND HAIR BY A VALIDATED LC-MS/MS METHOD

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A simple method for quantification of citalopram in mice plasma and hair was developed and validated using liquid chromatography tandem mass spectrometry (LC-MS/MS). The procedure involves a protein precipitation extraction of citalopram and desipramine (internal standard) with methanol from mice plasma. On the other hand, hair samples were incubated overnight with methanol at 45°C followed by  $\mu$ -SPE (OMIX Tip). The analysis was performed by resolving analytes in a Gemini® C18 column with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, at a flow rate of 250  $\mu$ L/min and with a total run time of 9 min. The mass spectrometer was operated in multiple reaction monitoring (MRM) by monitoring citalopram transition 325.3 $\rightarrow$ 109.0, and internal standard transition 267.3 $\rightarrow$ 72.2 for quantification. The qualifier transitions 325.3 $\rightarrow$ 83.1 and 267.3 $\rightarrow$ 190.8, respectively, were also monitored. Linearity was observed from 32.4 to 973.2 ng/mL and the limit of quantitation achieved was 32.4 ng/mL. Also, the intermediate precision, repeatability and accuracy were below the acceptance limits of 15%. This method was applied to plasma and hair samples that were collected from mice submitted to a treatment with citalopram for different days. The plasma concentration-time profile of citalopram showed a tendency to stabilize, approaching zero as samples were collected 24 hours after the last drug administration. In contrast, the concentration-time profile in hair increased over the period of 30 days.

## PROTEIN EXPRESSION LEVELS IN THE PRE-FRONTAL CORTEX OF MICE CHRONICALLY EXPOSED TO HALOPERIDOL

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Haloperidol was one of the first antipsychotics to be introduced and it is widely used in the treatment of patients with schizophrenia and related diseases for its high efficacy against positive symptoms, such as hallucinations and delusions. Nonetheless it has been associated with severe side-effects (as extrapyramidal side-effects, sedation and in extreme cases neuroleptic malignant syndrome).

Haloperidol has strong affinity for D2 receptors and a slow dissociation rate from these. A low dose of the drug is expected to rapidly block 60-80% of the D2 receptors, however up to 5 times more is used in clinical practice in order to produce the antipsychotic effect, which has a graduate and time-dependent onset of efficacy. This way, it has been difficult to define the mechanism of action of this drug, and understanding it may involve the study of long-term effects, not only on neurotransmitters and their receptors, but also on molecular pathways and their modulation which translate haloperidol action on neuronal activity.

The brain proteome is inevitably influenced by drug treatments, so in this study two different mass spectrometry proteomics approaches (label-free SWATH quantification and stable isotope labelling (iTRAQ)) were used to investigate differential quantitative protein expression in the rodent cortex, following chronic exposure to haloperidol. In order to increase the proteome coverage a subcellular fractionation was performed, using an ultracentrifugation step, giving rise to two distinct fractions: membrane-enriched and soluble protein fractions. The membrane-enriched fraction was processed using iTRAQ labelling for relative quantification and afterwards completed by SWATH-MS quantitative analysis. The soluble fraction was also processed by SWATH quantitative analysis.

With the proposed analysis, the quantification of over 2,000 proteins was achieved with about 60% of the identified proteins in the membrane-enriched fraction being annotated as “membrane”. Moreover, the use of two quantitative proteomic approaches (iTRAQ and SWATH) proved to be valuable and complementary.

From all the quantified proteins, and after applying statistical and quality filters, a subset of differentially expressed proteins was identified. More specifically, a gene ontology and molecular pathway analysis revealed that haloperidol leads to energy metabolic shift, and also modulates the glutamatergic and gabaergic synapse, as well as the synaptic vesicle cycle in the brain. Also the alterations of some signalling pathways are implicated, as in the example of altered ERK1/2 validated expression values.

In summary, in this study the alterations in the pre-frontal cortex of rodents upon chronic exposure to haloperidol were evaluated, and are being correlated for the potential understanding of the mechanisms of action of this drug. The results will highlight possible candidates for the study of the pathophysiology of psychosis and give clues about the long-term mechanism of action of the antipsychotic haloperidol. Moreover, these results aim to contribute with information to simplify the distinction between drug-related alterations from the ones intrinsic to the diseases, especially in postmortem brain studies.

**TOXICITY OF SYNTHETIC CANNABINOIDS IN NEURONAL, HEPATIC AND CARDIAC IN VITRO MODELS**

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The advent of new psychoactive substances (NPS) in Europe is one of the main concerns of European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Among the NPS, synthetic cannabinoids (SC) stand out as the most represented in the new drugs appearing in the European market every year. SC are extremely potent agonists of cannabinoid receptors, but the cell toxicity of these drugs is virtually unknown. Thus, the main aim of this work is to investigate the cell toxicity of synthetic cannabinoids.

To achieve this goal, the toxicity of five of the most consumed synthetic cannabinoids, AB-FUBINACA, JWH 122, 5-fluoro PB-22, THJ 2201 and XLR11, was evaluated in in vitro models, at concentration of 62.5  $\mu$ M - 2 mM, for 24 hours. A human neuroblastoma cell line (SH-SY5Y), a human hepatocellular carcinoma cell line (HepG2) and an embryonic rat heart cell line (H9c2) were used to evaluate neuronal, hepatic and cardiac toxicity, respectively. Mitochondrial activity of living cells was evaluated by MTT assay and the measurement of cellular protein content was performed using a Sulforhodamine B assay.

AB-FUBINACA, 5F-PB-22, JWH-122, THJ-2201 and XLR-11 were shown to reduce cell viability in the cardiac in vitro model. Additionally, AB-FUBINACA, 5F-PB-22, THJ-2201 and XLR-11 also decreased the cell viability in SH-SY5Y cell line, the neuronal in vitro model used. However, the synthetic cannabinoid AB-FUBINACA was the only one tested that reduced the cell viability in the hepatic in vitro model

In conclusion, the adverse effects reported after the consumption of synthetic cannabinoids are probably not only induced by the effect of drugs per se, but through their effects in peripheral organs. Thus, further mechanistic studies are needed to unravel the mechanism underlying the toxicity of these new psychoactive drugs.

## HYPOTHALAMIC LET-7 MICRORNA MODULATION PREVENTS OBESITY IN MICE

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Obesity causes cellular injury in the hypothalamus, a region of the brain responsible for maintenance of body homeostasis and feeding behaviour. This injury includes neuroinflammation, cell death and impaired neurogenesis [1]. It has been reported that inhibition of hypothalamic inflammation in obesity results in improved hepatic and adipose tissue metabolic parameters [2].

Let-7 microRNAs are putative candidates for a central anti-obesity therapy because they attenuate inflammation (in macrophages), control peripheral glucose homeostasis and promote neuronal differentiation of stem cells [3].

Therefore, the aim of this work was to investigate if let-7 microRNA modulation in the mouse hypothalamus could prevent the metabolic and hypothalamic alterations induced by obesity. For that, we over-expressed let-7 microRNA or control microRNA (miR-neg) in the mouse hypothalamus using lentiviral vectors and exposed the mice to high-fat diet (HFD) for 7 weeks. Metabolic parameters evaluated include: body weight, food consumption, glucose tolerance, serum cholesterol and triglycerides, adipose tissue weight and hypothalamic alterations (neuroinflammation and neurogenesis).

Mice with hypothalamic let-7 overexpression (HFD+let-7) gained weight at a slower rate than control mice (HFD+miR-neg), had decreased total caloric intake and showed less body fat accumulation. Moreover, HFD+let-7 mice had improved performance in the glucose tolerance test and presented decreased serum levels of cholesterol and triglycerides when compared to control HFD+miR-neg mice. At the hypothalamus, let-7 lentiviral overexpression could restore the let-7 expression levels reduced with HFD consumption. Moreover, HFD+let-7 mice showed decreased expression levels of inflammation mediators (IL-1 $\beta$ , IL-6) but presented no alterations in the expression levels of neurogenesis markers evaluated.

In conclusion, let-7 modulation was able to prevent the development of central and peripheral alterations characteristics of obesity in mice in HFD regime.

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## FUNCTIONAL GENOMICS ANALYSIS TO GATHERING EVIDENCE FOR m.7486G>A MUTATION PATHOGENICITY

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More than 20 variants, in 13 different mitochondrial transfer RNAs, are reported as a genetic cause of a Chronic Progressive External Ophthalmoplegia (CPEO) phenotype, characterized by slowly progressive paralysis of the extraocular muscles.

The novel m.7486G>A (*mt-TS1* gene) alteration was detected in a patient with CPEO diagnosis, by automated sequencing at the Biochemical Genetics Laboratory (LBG, CNC-Faculty of Medicine, Coimbra). According to *in silico* analysis using bioinformatics tools, this alteration is probably pathogenic.

In order to understand the functional impact of the novel alteration in the pathophysiology of the disease, some genetic, biochemical and functional studies were performed.

First, the sequence variation was confirmed by Next Generation Sequencing (at the Baylor College of Medicine, Houston) and by pyrosequencing at Mitochondrial NCG Diagnostic Laboratory, Newcastle. It is present in muscle, lymphocytes and fibroblasts with 50%, 4% and 10% of heteroplasmy, respectively. The mitochondrial respiratory chain activity was measured and a decreased activity in several complexes was detected, being the muscle the most affected tissue. The presence of COX-deficient fibres in this tissue allowed to perform single fibre studies, the gold standard method to prove the pathogenicity of mitochondrial transfer RNA alterations. It was also possible to confirm that the alteration segregates with COX-deficient fibres. Besides, functional studies were performed in patient's and controls' fibroblasts to evaluate metabolic changes. The results obtained by fluorimetry suggest mitochondrial membrane depolarization and increased superoxide production inside mitochondria. The mitochondrial ATP levels, the basal respiration and the maximal respiratory capacity are decreased and the glycolysis and the glycolytic capacity are increased showed by the Seahorse Bioscience analysis. Moreover, the electron microscopy revealed that patient's fibroblasts present abnormal intracellular structures.

The results suggest mitochondrial dysfunction in patient's fibroblasts and segregation of the mutation in COX-deficient muscle fibres. These facts highlight the high probability that the m.7486G>A variant is related to the pathogenicity of the disease.

## GLUCOSE AND LIPID METABOLISM ARE IMPAIRED IN EPICARDIAL ADIPOSE TISSUE FROM HEART FAILURE PATIENTS, WITH OR WITHOUT DIABETES

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Type 2 diabetes mellitus is a complex metabolic disease and cardiovascular disease is a leading complication of diabetes. Epicardial adipose tissue surrounding the heart displays biochemical, thermogenic and cardioprotective properties. However, the metabolic crosstalk between epicardial and the myocardium is largely unknown. This study sought to understand epicardial adipose tissue metabolism from heart failure patients, with or without diabetes. We aimed to unravel possible differences in glucose and lipid metabolism between freshly isolated human epicardial and subcutaneous adipocytes and elucidate the potential underlying mechanisms involved in heart failure. Insulin-stimulated <sup>14</sup>C-glucose uptake and isoproterenol-stimulated lipolysis were measured in isolated epicardial and subcutaneous adipocytes. The expression of genes involved in glucose and lipid metabolism was analyzed by reverse transcription polymerase chain reaction in adipocytes. In addition, epicardial and subcutaneous fatty acid composition was analyzed by high-resolution, proton nuclear magnetic resonance spectroscopy. The insulin fold induction in glucose uptake was significantly decreased ( $p=0.006$ ) in epicardial (median=5.83) compared to subcutaneous adipocytes (median=8.06). Moreover, a significant ( $p<0.001$ ) decrease in the isoproterenol-stimulated lipolysis was observed in epicardial (median=234.76) compared to subcutaneous adipocytes (median=737.50), and it was strongly correlated with lipolysis, lipid storage and inflammation-related gene expression. Moreover, the fatty acid composition of these tissues was significantly altered by diabetes. These results emphasize a potential metabolic difference between both fat depots in the presence of heart failure, and highlight epicardial fat as a possible therapeutic target, *in situ*, in the cardiac microenvironment.

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**EARLY SKIN AGING IN DIABETES IS CAUSED BY A DECREASE IN CB1 CANNABINOID RECEPTOR EXPRESSION**

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**Background:** The G protein-coupled metabotropic cannabinoid receptor type-1 (CB1R) is a major regulator of metabolism, growth and inflammation. Yet, its potential role in the skin is little understood. We found that CB1R expression was decreased in diabetic mouse skin. This prompted us comparing the expression of CB1R and other factors of inflammation and regeneration among the four groups of diabetic (DM) or sham Wild Type (WT) and CB1R knockout male mice (CB1RKO).

**Methods:** We quantified by q-RT-PCR markers of proliferation, inflammation, angiogenesis, oxidative stress, collagen (COL1A2, COL3A1) in the skin of wild-type (WT) control, WT streptozotocin (STZ)-induced diabetic mice and CB1R KO mice. We stain the skin collagen with trichrome masson. Also, we measure the levels of reactive oxygen species (ROS) in skin and the macrophage phenotype, M1 and M2, by immunohistochemistry.

**Results:** We found that CB1R expression is decreased in the skin of diabetic mice. The expression of collagen is impaired in diabetic and CB1KO mice, suggesting an early ageing process. The absence of CB1 receptors augmented the expression of several inflammatory markers interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), tumor necrosis factor-alpha (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1) and metalloproteinase-9 (MMP9). The ratio M1/M2 macrophage and the ROS levels were significantly higher under diabetic conditions and in CB1 mice, which are consistent with the decrease in the antioxidant capacity of the skin, particularly a decrease in Sod1, catalase and heme-oxygenase1 (Hox1) expression.

**Conclusions:** Our results indicate that the lack of CB1R impairs the expression of markers involved in the control of inflammation and tissue regeneration. These lead to accelerated skin ageing by the increased production of reactive oxygen species, a decrease in the antioxidant defenses and a higher pro-inflammatory environment. We conclude that the decreased CB1R expression may be a major pathologic mechanism in accelerated skin ageing in diabetes.

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## QUANTIFYING TRIGLYCERIDE FUTILE CYCLING WITH DEUTERATED WATER $^2\text{H}$ NMR ANALYSIS OF GLYCEROL $^2\text{H}$ ENRICHMENT

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Futile cycling” between triglyceride and fatty acids has been proposed as a mechanism for energy dissipation in living organisms. Our laboratory has developed a method for quantifying this rate of cycling using deuterated water ( $^2\text{H}_2\text{O}$ ) to label triglycerides. This method was based on the analysis of  $^2\text{H}$ -enrichment in the glyceride moiety by  $^2\text{H}$  NMR. The main goal of this Master dissertation was to develop an improved method for analysing  $^2\text{H}$ -enrichment in this site, since with existing triglyceride analysis, the glyceride  $^2\text{H}$  NMR signals are very broad and difficult to quantify due to their restricted motion. We therefore developed protocols to quantitatively transesterify triglycerides yielding free glycerol, whose  $^2\text{H}$  NMR signals are narrower and better resolved compared to those of the intact triglyceride. Since the glycerol  $^2\text{H}$  NMR analysis was performed in water, we tested hexafluoroacetone, an alternative fluorine lock compound to the hexafluorobenzene currently in use – but which is not water soluble.

We applied this analysis to quantify triglyceride/fatty acid futile cycling in adipose tissue of two mice strains (AJ mice - models of obesity-resistant- and B6 mice - models of obesity-prone animals) that were exposed to 7 days of cold in order to stimulate adipocyte futile cycling and heat generation pathways.

Following optimization of the transesterification protocol, we were able to obtain average glycerol yields of 80% that of the starting triglyceride material indicating that this approach was suitable for preservation of sample mass for effective NMR analysis. The  $^2\text{H}$  NMR spectrum of triglycerides shows two different peaks, one corresponds to the deuterium labelling in the carbon 2 of glyceride moiety and the other represents deuterium labelling in carbons 1 and 3 of the same moiety. The linewidths at half height for each peak are 19,46 Hz and 14,23 Hz, respectively. After the transesterification process, the  $^2\text{H}$  NMR spectrum of isolated glycerol shows 3 distinct peaks, one still corresponds to the deuterium labelling in the carbon 2 and two others corresponding to the prochiral *R* and *S* hydrogens bound to carbons 1 and 3. In this analysis, the linewidths at half height are 3,41 Hz, 3,68 Hz and 3,85 Hz, respectively. These narrower  $^2\text{H}$  NMR signals resulted in more precise measurements of triglyceride glyceryl  $^2\text{H}$  enrichment.

For the AJ strain, the fractional turnover rate of triglyceride glycerol, expressed in percent, were  $3,24 \pm 0,88\%$  and  $16,62 \pm 2,63\%$  ( $P < 0,001$ ) for the control and 7 days cold exposure, respectively. For the B6 strain fractional turnover rates of  $3,63 \pm 1,38\%$  and  $10,03 \pm 2,29\%$  ( $P < 0,001$ ) were obtained for control and 7 days cold exposure, respectively. Thus, AJ mice had significantly higher triglyceride turnover at 7 days cold exposure compared to B6 ( $P < 0,001$ ). This may be related to the reduced metabolic efficiency of AJ versus B6 mice that in turn may better protect the AJ strain against diet-induced obesity.

# KAEMPFEROL: NATURAL PLURIPOTENCY ENHANCER OR STEM CELL KILLER – JUST A QUESTION OF BALANCES

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Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid which has several beneficial properties once it acts for example as an antioxidant compound that can impact proliferation, apoptosis and cell cycle in cancer cells. Nevertheless, its possible effects in embryonic stem cells have not been addressed. Embryonic stem cells (ESC) have the ability to self-renew and to differentiate into cells from all the three embryonic germ layers with potential applications in regenerative medicine and toxicology.

Herein we show that exposure of murine ESC (mESC) to high concentrations of kaempferol leads to a decrease of cell number resulting in smaller morphologically pluripotent colonies. Nonetheless, small concentrations of this flavonoid increase pluripotency in mESCs. Mitochondrial membrane potential and mitochondrial mass are not affected by kaempferol, but a dose-dependent apoptosis induction occurs, which seems to be, at least in part, due to increase of mitochondrial reactive oxygen species. Nevertheless, although mESC colonies are smaller and have increased apoptosis, pluripotency seems to be unaffected by higher kaempferol concentrations, implying a possible toxic side effect, without detrimental effects on pluripotency. Moreover, mESC differentiation is impaired by kaempferol, which is not related to apoptosis induction.

Our results show that low concentrations of kaempferol are beneficial for pluripotency, while inhibiting differentiation of mESCs. However, high concentrations of this compound induce apoptosis which seem to be related to an increased in mitochondrial ROS levels. Therefore, natural molecules, as kaempferol in our study, can be used to benefit stem cell culture by regulating pluripotency maintenance or differentiation, if and when proper concentrations are determined, thus avoiding the use of non-natural and non-physiological chemical engineered factors in culture conditions.

## LACK OF LYMPHOCYTES IMPAIRS SKIN WOUND HEALING

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**BACKGROUND:** Diabetic foot ulceration is one of the major diabetic complications (1). In normal tissues, wound healing is characterized by efficient inflammatory cell recruitment in response to a variety of chemokines, but in diabetic patients there is an impaired leucocyte function, dysfunctional inflammatory cell infiltration and inflammation, and inadequate migration to the wound among others alterations (2, 3). Substance P is released by cutaneous neurons and modulates the function of inflammatory cells, helping the wound healing (4). The aim of this study was to investigate the inflammatory phase of diabetic wound healing and the effect of Substance P using the knockout mice Balb/c/Rag2<sup>-/-</sup>IL2R<sup>-/-</sup> lacking the B,  $\alpha\beta$  T and NK cells(5).

**METHODS:** Two 6-mm diameter full-thickness wounds were created with a biopsy punch in Balb/c wild type (WT) and Balb/c/Rag2<sup>-/-</sup>IL2R<sup>-/-</sup> (Rag2 knockout - KO) mice under anesthesia. The wounds were treated or not with substance P during 10 days in some animals from each group, compounding the group d3+SP. Wound healing was evaluated daily up to 10 days by acetate tracing. The wound area was quantified using ImageJ software. After the experimental period (3 and 10 days) the animals were anesthetized and sacrificed by cervical displacement and around 2 mm of tissue and skin surrounding the wound were harvested. For mRNA analysis, we used 6 groups of animals: WT: d0, d3 and d3+SP, and KO: d0, d3 and d3+SP. Total RNA was extracted from skins, and the mRNA expression of TNF, substance P (SP), neurokinin receptor 1 - NRK1 (receptor of SP), and KC was determined by Real-time qPCR.

**RESULTS:** The rate of wound closure was significantly delayed at day 3 in KO mice (102.79%±3.93% vs. 81.79%±4.05%, p<0.001). Both groups presented their wounds closed by 50% at day 7 and 80% by day 10 after wounding. The final wound healing process was similar in both control and KO mice. The mRNA expression of TNF in WT was increased in day 3 compared to day 0 (p<0.001). KO mice presented a decreased in TNF expression (25%, p<0.05) in day 0 and day 3 compared to WT, however the SP treatment in KO mice improved this expression, without alteration between WT and KO mice in the day 3. KC expression in day 0 and day 3 was reduced (70%, p<0.05, and 82%, p<0.005) in KO mice compared to WT, and SP treatment did not change this condition. SP mRNA expression was unchanged between WT and KO in day 0 and 3, but it was reducing along the time. In WT, NRK1 expression was increased (85%, p<0.05) in day 3 compared to day 0, while in KO it was reduced (70%, p<0.05) in day 3 compared to day 0. NRK1 expression was increased (6.5 times, p<0.05) in KO in day 0, but it was reduced (60%, p<0.05) in day 3 compared to WT. However similar NrK1 levels were maintained in KO and WT with SP treatment.

**CONCLUSION:** The complete lack of lymphocytes results in a significant impairment of the earlier phase of the wound healing process, particularly during the inflammatory phase (day 3) indicating their significant role in the early healing process. The wound closure is similar between wild type and Rag2 knockout mice, presenting closure around 50% at day 7 and 80% at day 10, after wounding. Knockout mice present a decreased mRNA expression of TNF- $\alpha$  and KC in the wound site, however Substance P treatment seems to increase inflammatory marker expression at the wound site.

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## ATAXIN-2 IN THE HYPOTHALAMUS: A NOVEL REGULATOR OF ENERGY BALANCE AND METABOLISM

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Ataxin-2 is a polyglutamine protein implicated in biological processes such as RNA metabolism and cytoskeleton reorganization, however its functions are not yet completely understood. In its mutated form (above 31 CAGs repeat) ataxin-2 is the causative protein of the autosomal dominant genetic neurodegenerative disease, spinocerebellar ataxia type 2 (SCA2). On the other hand, ataxin-2 knockout mice are obese and present several metabolic alterations (Kiehl et al., Biochem Biophys Res Commun 2006; Lastres-Becker et al., Hum Mol Genet. 2008). Moreover, polyphagia has been reported in an Egyptian family with SCA2, suggesting that ataxin-2 mutation may cause metabolic alterations (Abdel-Alleem & Zaki, J Neurol. 2008).

The aim of the present work was to unravel the role of hypothalamic ataxin-2 on metabolism regulation. For this purpose we used lentiviral vectors to silence and overexpress ataxin-2 in the arcuate nucleus of hypothalamus of C56BL/6 mice. Mice were fed an *ad libitum* rodent chow diet throughout the duration of the study, food consumption and animal weight were assessed every two days. Before the end of the study, insulin tolerance (ITT) was evaluated.

Food intake was not different between the three groups of animals (control, hypothalamic ataxin-2 overexpression and hypothalamic ataxin-2 silencing). Nevertheless, mice with hypothalamic ARC ataxin-2 overexpression showed a significant lower body weight gain relative to control mice. Furthermore, mice with ataxin-2 silencing in hypothalamic ARC presented higher body weight gain, more white adipose tissue, less brown adipose tissue, higher levels of Neuropeptide Y (NPY) mRNA and a delayed response to insulin, compared to control group.

These results suggest that hypothalamic ataxin-2 contributes to the regulation of energy balance and metabolism.

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## PREDIABETES INDUCES HYPERTRIGLYCERIDEMIA BY ENHANCING LIVER LIPID BIOSYNTHESIS IN A RAT MODEL

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**BACKGROUND:** Elevated plasma triglycerides (TG) is emerging as an independent risk factor for Type 2 diabetes (T2D), metabolic syndrome [1,2], hepatic steatosis and pancreatitis [3]. Moreover, lipotoxicity has recently been shown to be an important risk factor underlying the pathogenesis of prediabetes [4]. In fact, hypertriglyceridemia is often accompanied by increased concentrations of triglyceride-rich lipoproteins (TRL) and this phenotype is predominantly seen in disorders characterized by insulin resistance (IR), namely T2D [5] and prediabetes. Actually, IR drives the increased flux of free fatty acids (FFAs) to the liver and stimulates TRL synthesis and secretion by this organ [6]. Consequently, excessive hepatic ectopic deposition of TG may contribute to IR, inflammation and organ dysfunction [7]. As the underlying cause of dysregulated metabolism of TRL is IR, our goal was to reveal the molecular mechanism underlying the hypertriglyceridemia observed in a prediabetic rat model, previously characterized as having IR [8].

**METHODS:** Two groups of 16-week-old Wistar rats were tested during a 9 week protocol: high sucrose (HSu) diet group (n=30) – rats received 35% of sucrose in the drinking water – versus the vehicle, water alone as the control group (n=32). The animal model was characterized in terms of body weight (BW), insulin resistance, glycemic, insulinemic and lipidic profiles. The following parameters were assessed to evaluate hepatic function: liver weight/BW ratio and analysis of liver function by biochemical parameters, namely, alanine transaminase (ALT) and aspartate transaminase (AST). Also, we assessed hepatic TG biosynthesis signaling cascade and isoproterenol-stimulated lipolysis in epididymal adipose tissue, which could increase the flux of FFAs to the liver, providing substrate for hepatic TG synthesis.

**RESULTS:** The HSu-treated rats presented impaired glucose tolerance, accompanied by hyperinsulinemia and insulin resistance, confirming this rat model as prediabetic. Furthermore, although hypertriglyceridemia was observed, obesity was absent and BW was normal. Regarding the impact of the HSu diet on the liver, our results indicated that HSu diet might be associated with initial hepatic changes, as suggested by the increased liver weight and by the remarkable decrease in ALT levels. Also, we observed increased acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) protein expression in the livers from HSu-treated rats, which might stimulate hepatic TG synthesis and secretion into the bloodstream. However, lipolysis was not significantly higher compared to the control group.

**CONCLUSION:** This animal model of prediabetes/IR could be an important tool to evaluate the impact of HSu diet on early hepatic dysmetabolism, without confounding factors such as obesity. Increased expression of ACC1 and FAS in the liver seems to be the best molecular markers of ectopic TG deposition in the liver, leading to increased liver weight/BW ratio and decreased hepatic function. In the near future, we expect that these new insights might contribute to the disclosure of efficient therapeutic strategies to ameliorate the natural course of hypertriglyceridaemic prediabetes and prevent or stop the progression to DM.

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## PHARMACOLOGICAL MODULATION OF MUTANT ATAXIN-3 AND ITS POTENTIAL THERAPEUTIC EFFECT IN MACHADO-JOSEPH DISEASE

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### ABSTRACT

Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is described as the most common ataxia worldwide. It is associated with the expansion of a (CAG)<sub>n</sub> tract in the coding region of the causative gene MJD1. This abnormal over-repetition is translated into an expanded polyglutamine tract within ataxin-3 protein, resulting in severe clinical features leading to neurodegeneration and premature death. Despite important progresses, the mechanisms accounting for neuronal degeneration are still largely unknown and there is no available treatment. In this project we aim evaluate a pharmacological therapeutic approach for MJD, using an adenosine analogue, cordycepin implicated in several biochemical and molecular processes.

*In vitro* experiments allowed us to find an accurate concentration and treatment time, as well as to detect the beneficial effect of the drug in reducing the levels of mutant protein. With the lentiviral model (based in the local expression of Atx3-72Q), the neuropathological features were evaluated and it was detected a reduction in the number of inclusions accompanied by a decrease in neuronal loss. The transgenic model (cerebellar expression of Atx3-69Q) allowed us to detect a rescue of the motor deficits phenotype upon 6-weeks treatment with cordycepin.

Altogether these results suggest that cordycepin has the ability to ameliorate some characteristic features of MJD. Taking this into account and the fact that a pharmacological method facilitates the application of the approach to MJD clinics in a very short time frame, cordycepin could play a promissory role in the treatment of MJD or even be extended to other disorders, as it is generally accepted that spinocerebellar ataxias share common pathogenesis.

**Keywords:** Machado Joseph disease (MJD)/Spinocerebellar ataxia type 3 (SCA3), Cordycepin/3'deoxyadenosine

## ENHANCED EFFICACY OF TARGETED SYNERGISTIC DRUG COMBINATIONS AGAINST NUCLEOLIN-OVEREXPRESSING OVARIAN CANCER CELL LINES

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Ovarian cancer is the fifth major cause of cancer death in females. The high mortality rate is associated with a late-stage diagnosis and tumor recurrence after platinum therapy. There is evidence that a putative population of tumor cells, called cancer stem cells (CSC), is involved not only in recurrence but also in tumorigenicity, metastasis and drug resistance [1]. Nevertheless, the acknowledgment that CSC may originate from non-stem cancer cells, interconverting through an Epithelial-to-Mesenchymal Transition-mediated process has turned these cell subpopulations into two relevant therapeutic targets. Another important aspect to consider is the fact the PI3K/AKT/mTOR pathway is essential for CSC proliferation and survival and it is often over activated in ovarian cancer. One of the strategies to reach these different tumor cell populations, relies on the combination of conventional chemotherapeutic drugs (as tumor debulking agents targeting non-stem cancer cells) with sphingolipids targeting CSC (at the level of PI3K/Akt/mTOR) [2].

We have previously developed a lipid-based nanoparticle containing doxorubicin and functionalized with the nucleolin-binding F3 peptide. Nucleolin overexpression has been demonstrated on the surface of both breast cancer (putative CSC and non-stem cancer cells) and endothelial cells [3]. The F3 peptide-targeted pH-sensitive lipid-based nanoparticle was recently modified to contain a synergistic drug combination of a sphingolipid and doxorubicin [4]. Following the promising results in breast cancer, the aim of this work was to test the potential of this strategy against ovarian cancer.

*In vitro* studies with (bulk) ovarian cancer cell lines demonstrated a significant improvement in cellular association (including internalization) of the F3 peptide-targeted liposomes (F3-L), relative to the non-targeted counterparts. Importantly, a similar pattern of association with the putative CSC enriched population was also observed. These results corroborated the marked increase of cytotoxicity enabled by the targeted drug combinations relative to F3 peptide-targeted liposomes containing only doxorubicin.

Taken together, these results pointed out the therapeutic potential arising from the intracellular delivery of the developed synergistic drug combination against ovarian cancer cell lines.

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## MIMICKING CALORIC RESTRICTION THROUGH MOLECULAR AND PHARMACOLOGICAL SIRT1 ACTIVATION ALLEVIATES MACHADO-JOSEPH DISEASE

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Machado-Joseph disease (MJD) also known as Spinocerebellar Ataxia type 3 (SCA3) is a neurodegenerative disorder characterized by an abnormal expansion of the CAG triplet in the *ATXN3* gene, translating into a polyglutamine tract within the ataxin-3 protein. MJD is a fatal and inherited disease without any treatment to cure or to retard the progression of the disease. Caloric restriction (CR) has neuroprotective properties and slows down the occurrence of age-dependent diseases. The mechanisms underlying these effects are intrinsically related to sirtuin 1 (SIRT1), a NAD<sup>+</sup>-dependent enzyme, with a deacetylase activity. The putative beneficial role of CR and SIRT1 on MJD progression was not investigated before.

We aimed at investigating the effects of CR on mouse models of MJD and at unravelling the role of SIRT1, evaluating its potential for the treatment of Machado-Joseph disease.

We used a transgenic MJD mouse model and a striatal lentiviral mouse model of MJD, as previously described (Simões et al., 2012; Torashima et al., 2008). MJD transgenic mice were submitted to 30% of CR diet or to an *ad libitum* diet. In order to explore the contribution of SIRT1 to CR effects, lentivirus encoding for SIRT1 (or H363Y as control) or for a shRNA targeting SIRT1 (or a control shRNA) were injected in the MJD mouse striatum in striatal lentiviral model of MJD. Furthermore, pharmacological activation of SIRT1 was performed through the intraperitoneal injection of mice with 10 mg/kg of resveratrol versus vehicle (25% DMSO in saline solution).

We evaluated the impact of caloric restriction in the phenotype and neuropathology of mouse models of MJD. We observed that caloric restriction dramatically rescues the motor incoordination, imbalance and the associated neuropathology in transgenic MJD mice, due to the amelioration of specific hallmarks. We also observed that such effects are majorly mediated by SIRT1, as caloric restriction reverts abnormal decrease of SIRT1 levels in transgenic MJD mice and these effects are blocked upon SIRT1 genetic silencing. Additionally, the reestablishment of SIRT1 levels in MJD mouse models, through gene delivery approach, significantly ameliorated neuropathology, reducing neuroinflammation and activating autophagy. Furthermore, the pharmacological activation of SIRT1 with resveratrol strongly reduced motor incoordination of MJD mice.

This study shows that molecular and pharmacological SIRT1 activation delays disease progression, in several MJD mouse models. Therefore, a therapy based on SIRT1 activation could provide important benefits to treat MJD patients.

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## ANTIVIRAL POTENTIAL OF NATURAL EXTRACTS

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### Background and aims

The discovery and development of new antiviral drugs are urgently needed, mainly for the treatment of high mortality/morbidity viral diseases, especially among immunocompromised patients. It is also imperative the discovery of new therapeutic tools to cope with the emergence of viral strains resistant to current antiviral therapies.

In recent years there has been an increasing interest in natural substances as a potential source of antimicrobial agents because of their several benefits, namely, their presence in nature, their multiple targets, minor side effects, low potentials to cause resistance and low cost.

The aim of this study was to assess the antiviral activity of 5 natural extracts.

### Methods

We used two models of viral infection, HEK-293T/Lentivirus (LV) and Caco-2/Coxsackievirus A12 (CVA12) for the screening of the antiviral potential of extracts from two olive varieties (CO) and (MO); and from algae of the Filo *Rhodophyta*, extracts T, FG, and OP.

Prior to antiviral assays the cytotoxicity of the used extracts was evaluated.

### Results

On the HEK-293T/Lentivirus model, all extracts induced a state of cellular resistance to infection and a virucidal effect. On Caco-2/Coxsackievirus A12 model, all extracts reveal potential as virucidal agents with the exception of FG extract; CO and MO extracts also reveal potential to protect cells against infection and OP extract it was also effective in controlling viral replication when added post-infection.

### Conclusion

These results suggest that all the extracts have an inhibitory effect on LV infection at an early stage and therefore can be candidates for prophylactic intervention; the same is true for CO and MO extracts in CVA12 infection. OP extract it may be a candidate for therapeutic intervention on CVA12 infection because exhibits inhibitory effect in viral replication.

## IS AGEING THE TRIGGERING FACTOR IN POLYGLUTAMINE INDUCED NEURODEGENERATION? A STUDY IN MACHADO-JOSEPH DISEASE MODELS

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Machado-Joseph disease (MJD), also known as Spinocerebellar ataxia type 3 (SCA3) is an autosomal dominantly-inherited neurodegenerative disorder characterized by the pathological expansion of CAG trinucleotide repeat in the *ATXN3/MJD1* gene, which confers a toxic gain-of-function to the mutant protein – ataxin-3, leading to neurodegeneration in specific brain regions (Nobrega and de Almeida, 2012). Until now no treatment able to modify the disease progression is available, despite the promising progresses made by our group and by others.

Ageing is the major risk factor for neurodegenerative disorders (Hung et al., 2010). While some ageing-associated changes occur in a programmed manner, others are stochastic and unpredictable (Kirkwood and Melov, 2011). An alternative to the analysis of ageing is the study of human genetic syndromes whose phenotypes show specific characteristics of human ageing, and Hutchinson-Gilford Progeria Syndrome (HGPS), a premature ageing syndrome, is one of those examples. It is caused by the improper processing of lamin A protein, result of a point mutation leading to the accumulation of the mutated form, termed progerin, in the nuclear envelope (Eriksson et al., 2003).

In this project we will investigate the role of ageing in polyglutamine-induced degeneration.

We observed that MJD patients' fibroblasts and the cerebellum of MJD transgenic mouse model contain lower mRNA and protein levels of zmpste24 (a protein highly associated with ageing mechanisms), compared to controls; These results might indicate an aged phenotype in MJD models.

The striatal lentiviral model of MJD, where the injection of viruses encoding for mutant ataxin-3 was associated with an induced accelerated ageing setting (through the overexpression of progerin), revealed an aggravation in MJD pathological features. It was possible to detect, through Immunohistochemical analysis, a significant increase in the loss of DARPP32, and a tendency to increase pyknotic nuclei in comparison with the control brain hemisphere.

Other ageing associated mechanisms are being pursued and we expect to clarify their role in MJD pathogenesis and age of onset. Moreover, stopping/delaying the mechanisms involved in normal or accelerated ageing may represent a novel approach for therapy or delaying the symptoms associated with MJD.

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## TREHALOSE REVERSES DISEASE PHENOTYPE OF A MOUSE MODEL OF MACHADO-JOSEPH DISEASE

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Machado–Joseph disease (MJD), also known as spinocerebellar ataxia type 3, is the most common of the dominantly inherited ataxias worldwide and is characterized by mutant ataxin-3 misfolding, intracellular accumulation of aggregates and neuronal degeneration. No treatment able to modify the disease progression is available. In this project, we will evaluate whether trehalose, a natural occurring alpha-linked disaccharide, could rescue the behavioral and neuropathological features of a transgenic MJD mouse model. MJD transgenic mice were orally treated with 2% trehalose solution for a period of 30 weeks. Motor behavior was measured at different time points during lifetime and neuropathological features were evaluated after sacrifice. We observed that trehalose treatment significantly improved the motor and coordination behaviour in stationary rotarod test, beam walking, swimming test and footprint analysis. Moreover, trehalose reduced the MJD-associated neuropathology of MJD transgenic mice, which presented less atrophy of cerebellum layers and a decrease in the size of protein aggregates in Purkinje cells, suggesting a prevention of neurodegeneration in the treatment group. In conclusion, this study supports the notion that trehalose could be an effective therapeutic agent for MJD disease.

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## IGY-TECHNOLOGY REVISITED

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The emergence of multidrug resistant bacteria is a major global threat and its evolution at alarming rates urges the implementation of different action plans, including the development of new antimicrobial drugs. Avian IgY antibodies hold significant potential to help controlling the spread of multidrug resistance.

In the last three decades there has been increasing interest in the IgY-technology, a concept that involves the recovery of IgY antibodies deposited in egg yolks of immunized hens and their subsequent use in passive immunotherapies against microbial and viral infections. IgY antibodies have been used for example to prevent infections of *Pseudomonas aeruginosa* in patients with cystic fibrosis or to suppress *Helicobacter pylori* infection in patients with gastric disorders.

IgY immunotherapy is recognized as a low toxicity treatment, therefore suitable for long term prophylaxis, particularly when prolonged exposure to the virulent pathogen cannot be avoided.

Economically, chickens are very attractive bioreactors, with a single hen substituting up to 12 rabbits in antibody production over one year. However the major drawback of the IgY-technology still persists: there is no cheap and efficient method allowing high recovery of pure IgY from egg-yolk.

Refining avian models to become the source of relevant antimicrobial biological drugs demands a synergistic effort between biomolecular and industrial production expertises.

We have been exploring the advantages and revisiting the limitations of IgY-technology, aiming to further develop alternative IgY production and purification methodologies. We have been focused on two avian models as antibody bioreactors: *Coturnix japonica* and *Gallus gallus*. The first is a fast-maturing bird, allowing rapid generation of multiple immunological repertoires against targets of interest; the second is the most industrialized egg-laying bird, thus supporting large-scale production of IgY-based drugs.

DERIVATION OF A HUMAN *IN VITRO* BBB MODEL FROM HIPSCS

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**Introduction:** The blood-brain barrier (BBB) is one of the most important barriers between blood and brain, being crucial for the maintenance of the neuronal microenvironment. Using BBB models is possible to make reliable predictions of drug-BBB interactions and also to study biological and pathological aspects of the barrier. Recently we have generated a stable and reproducible human *in vitro* BBB model derived from cord blood hematopoietic stem cells (Cecchelli et al., PLoS One 2014). The cells were initially differentiated into endothelial cells (ECs) followed by the induction of BBB properties by co-culture with bovine pericytes. The brain-like endothelial cells (BLECs) expressed tight junctions and transporters typically observed in brain endothelium and maintained the expression of most BBB properties for at least 20 days. However, so far, the derivation of disease models of human BBB has not been reported. Induced pluripotent stem cells (iPSCs) represent a promising source of BLECs with specific disease phenotypes such as Alzheimer, Parkinson and other neurodegenerative diseases. This is not currently possible using the human BBB system developed by us from human cord blood hematopoietic stem cells. Here, we describe a procedure to derive BLECs from hiPSCs using well-characterized progenitor cells. We further document the effect of extracellular matrix (ECM) and soluble factors in this inductive process, and we report their functional activity.

**Results and Discussion:** Vascular progenitor cells isolated from hiPSCs express high levels of CD31 (~ 91%) and GLUT-1 (~ 97%), moderate levels of claudin-5 (~ 41%), occludin (~ 25%) and ZO-1 (~ 57%), and no levels of P-gp. Immunocytochemistry results showed that the expression of occludin and claudin-5 is not entirely at cell junctions, disclosing an immature BBB phenotype. These cells were matured for 4 passages in specific cell media conditions and specific native decellularized matrices. Along the maturation process, there is an increase in the co-localization of the CD31 marker with some of the BBB markers (Claudin-5, ZO-1 and P-gp), suggesting a specification for the BBB phenotype. Functionally, these cells when cultured in the transwell systems, present relatively low permeability to Lucifer yellow ( $1.24 \pm 0.14 \times 10^{-3}$  cm/min), TEER values of  $55 \pm 0.58 \Omega \text{cm}^2$  and are able to generate a continuous monolayer presenting ZO-1 and claudin-5 in the cell membrane.

**Conclusion:** In this work we report a methodology to differentiate hiPSCs into ECs with BBB properties (BLECs). Our results further highlight the importance of specific soluble factors and neurovascular unit ECM in the differentiation of vascular progenitor cells.

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