

# Histology and Histopathology

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DE HISTOLOGÍA E INGENIERÍA TISULAR -  
V INTERNATIONAL CONGRESS OF  
HISTOLOGY AND TISSUE ENGINEERING*



# HISTOLOGY AND HISTOPATHOLOGY

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LOGROÑO, SPAIN, SEPTEMBER 12<sup>th</sup>-14<sup>th</sup>, 2013

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## PLENARY SESSIONS

### **P.1 MARIANO LÓPEZ MATEOS Y LA INTRODUCCIÓN DE LA TEORÍA CELULAR EN ESPAÑA.**

### **MARIANO LÓPEZ MATEOS AND THE INTRODUCTION OF CELL THEORY IN SPAIN**

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During the first half of the nineteenth century an important paradigm shift was carried out in the medicine and the natural sciences: the birth of the cell theory and its increasing influence in the concept and development of medicine. In the context of the first half of the nineteenth-century in Spain after the war of independence and the reign of Fernando VII, a difficult time for science in Spain, Mariano López Mateos (1800-1863), who was professor in the university of Granada, incorporates the basic concepts of the cell theory through his book "Treaty of Histology and Ovology", published in Granada in 1853, prior to what happens in other European countries. After Mariano López Mateos the histological studies will reach a high develop in Granada with the figure of Aureliano Maestre de San Juan, who became later in Madrid the first full professor of histology in Spain and the founder of the Spanish Histological Society.

## **P.2 TRANSPORTE LISOSOMAL. DESARROLLO DE ESTRATEGIAS TERAPÉUTICAS PARA EL TRATAMIENTO DE ENFERMEDADES INFECCIOSAS Y LISOSOMALES**

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El transporte de glicoproteínas a su destinación final depende exclusivamente del aparato de Golgi. La función del aparato de Golgi es glicosilar, seleccionar y enviar proteínas sintetizadas en el retículo endoplasmático a los lisosomas, al espacio extracelular o a la membrana plasmática. Las proteínas lisosomales incluyen enzimas hidrolíticas y activadores de esfingolípidos (SAPs), los que si bien no tienen funciones enzimáticas son requeridos en la degradación de esfingolípidos. La mayoría de estas proteínas son transportadas a los lisosomas por dos receptores de manosa-6-fosfato (MPRs). Las mutaciones que afectan la estructura y expresión de estos receptores, como así también de las enzimas hidrolíticas y de los activadores de esfingolípidos, resultan en un grupo de 40 enfermedades lisosomales conocidas como “Desórdenes de Almacenamiento Lisosomal”, (DAL), los cuales son causadas por la ausencia o actividad defectuosa de proteínas lisosomales. Los DAL producen una acumulación de metabolitos no degradados en los lisosomas. Recientemente identificamos en nuestro laboratorio un receptor alternativo a los MPRs, llamado “sortilina”, el cual se encuentra involucrado en el tráfico lisosomal del activador “*prosaposina*” y de las enzimas hidrolíticas “*catepsina D*”, “*catepsina H*” y “*esfingomielinasa ácida*”. La sortilina pertenece a una familia de proteínas emparentadas con el receptor lisosomal “Vps10” de la levadura. Nuestro laboratorio también ha identificado una región de 23 aminoácidos en el segmento “C-terminal” de la prosaposina que interactúa con la sortilina. La eliminación de este segmento inhibe el transporte de la prosaposina a los lisosomas e induce un DAL característico de la “*Leucodistrofia Metacromática*.” Dado a que la mayoría de los agentes infecciosos entran en las células a través de los lisosomas usando la vía endocítica, nuestro descubrimiento puede ser útil en la eliminación de patógenos en el compartimento lisosomal. En esta disertación se presentarán evidencias estableciendo la validez de este concepto y se discutirá el potencial terapéutico de nuestro hallazgo en el tratamiento alternativo de las enfermedades infecciosas y en el tratamiento de los Desórdenes de Almacenamiento Lisosomal.

Esta investigación fue efectuada gracias a un subsidio del NSERC, Canadá.

### P.3 WE AGE WITH OUR ELASTIC FIBERS – NEW INSIGHTS INTO MOLECULAR CHANGES

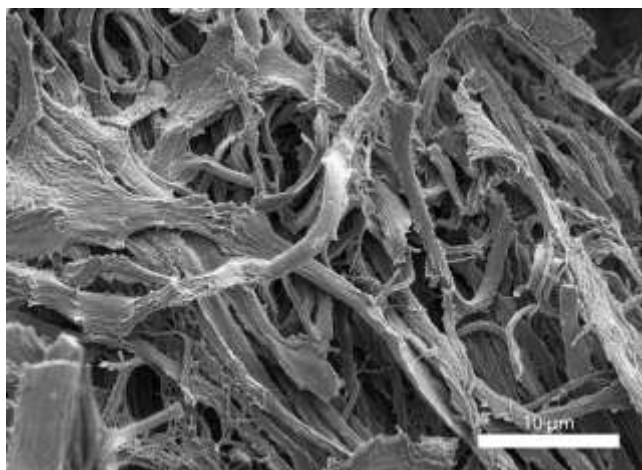
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The ability to undergo elastic deformations is vital to the function of many human tissues such as lung, blood vessels or skin. Depending on physiological function, differently organized fiber networks are formed inside the extracellular matrix during development. Once deposition of the fibers has been completed, the fibers have to maintain their function



Disintegrated elastin fibers of a 90-years old woman.

throughout the lifetime of the individual without repair or replacement. Damages, which may occur as a consequence of processes such as enzyme dysregulation, pathological conditions but also aging, are irreversible and result in a loss of elasticity and severe diseases. To understand the structural changes of elastin during these processes, it is necessary to gain insight into the morphological and molecular constitution. Thus, we developed methods that facilitate the isolation of highly purified and intact elastin fibers from human punch biopsies. These

biopsies were derived from cartilage, skin, aorta and intervertebral disc of differently aged or diseased individuals. Mass spectrometric and electron microscopy techniques revealed age- and tissue-related differences in the peptide patterns of elastins.

The identification and quantification of such markers enables the investigation of elastolytic abilities of proteases to degrade elastin at particular residues and thus allow conclusions to be drawn about the integrity and molecular characteristics including PTMs of different elastins. Furthermore, marker peptides may be used as “molecular clocks” to determine the biological age of tissues or their degree of degeneration. This novel approach permits the recognition of potential changes and modifications of elastin as well as other long-lasting ECM proteins.



## A. Histopathology:

### A.1 HISTOLOGY FOR MEDICAL STUDENTS

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Histology is a fundamental subject to understand the functioning of the different organs and systems. It is, therefore, the essential basis for the study of physiology. In addition, in the medical degree (and also dental or veterinary degrees) Histology is a core subject for the study of pathology.

First year medical students are eager to have a first contact with clinical medicine, and Histology should facilitate this first step. Hence, it is essential that the professor understands, since the first day, that he or she should teach Medical Histology highlighting everything that is important as introduction to pathology. In our experience this approach greatly motivates students. This is already reflected in several texts of Histology, which have included issue boxes with introductions to pathology and questions about clinical cases in each chapter.

In modern medicine, histopathology continues to be a fundamental pillar of clinical practice. The development of Immunohistochemistry in the last three decades has revolutionized histopathological diagnostics (a “brown revolution” has been talked about), to the point that at present time it would be impossible to practice pathology without the help of this technique. Immunohistochemistry facilitates the differential diagnostic between different tumour types. But also, and this is most important, between *in situ* and infiltrating carcinomas and even between benignancy and malignancy in some tumour types. Moreover, the development of specific cell markers was an important step in the study of metastasis of unknown origin. Immunohistochemistry even allowed for the discovery of new entities, i.e. gastrointestinal stromal tumour (GIST) or Ki1 lymphoma (nowadays known as anaplastic large cell lymphoma). With the development of predictive markers for the response to new cancer treatments, Immunohistochemistry has become a key factor in the selection of patients for the use of targeted therapies (personalised medicine); which in cases such as HER2 it signified a *before and after* in the prognosis of this aggressive subtype of breast cancer (and now in gastric adenocarcinoma, as well), representing one of the most spectacular advances of medical oncology in recent times.

In the presentation we will discuss some of these applications of Immunohistochemistry as a bridge between Histology and pathology; which will serve as an incentive to our students to value the importance of the study of Histology in their medical training.

## **A.2 ADRENOMEDULLIN REGULATES BRONCHIOLAR REPAIR FOLLOWING CLARA CELL-SPECIFIC INJURY**

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Adrenomedullin (AM) is a ubiquitous peptide that is expressed in bronchio-alveolar epithelial cells, alveolar macrophages, endothelium, and smooth muscle cells of the lungs. Although previous studies have shown that AM promotes lung angiogenesis, alveolar development, and parenchyma repair, very little is known about the role that AM may play in airway epithelium repair following injury. Clara cells are non-ciliated, secretory bronchiolar epithelial cells that serve to detoxify xenobiotics and oxidant gasses, control the extent of inflammation, and participate in mucociliary clearance of environmental agents. A mouse model of Clara cell-specific airway epithelial damage involves intraperitoneally administered naphthalene and was used in this study. Naphthalene is an environmental toxicant with genotoxic and carcinogenic potential. This compound is also found in the particulate phase of cigarette smoke. Naphthalene is metabolized in the lung generating reactive intermediates which deplete glutathione, covalently bind to proteins, and cause necrosis in Clara cells. To investigate whether AM is involved in the regeneration of the bronchiolar epithelium, we administered naphthalene to both wild-type and Clara cell-specific AM conditional knockout mice.

Both wild-type and knockout female C57/BL6 mice were injected with 200 mg/kg naphthalene and culled at time points 0, 2, 5 and 14 days post injection. Mice were given intraperitoneal injections of bromodeoxyuridine (BrdU) 2 hours before sacrifice to track cell proliferation during repair. The lungs were dissected, processed, embedded in paraffin, and stained with haematoxylin and eosin for histological analysis. RNA was isolated from frozen lungs and gene expression was analyzed by quantitative RT-PCR. Triple-label immunofluorescence staining of lungs for Clara cell specific protein, ciliated cell marker (Fox-J1), and BrdU were counterstained with DAPI as a nuclear stain. The images were captured with confocal microscopy.

Histomorphological analysis of captured images revealed significant delay in Clara cell renewal in the bronchiolar epithelium of knockout mice versus wild type mice. These results were also confirmed by mRNA levels, being lung CCSP mRNA expression significantly reduced in knockout mice after naphthalene challenge at 2 and 14 days.

Taken together, our results demonstrate that AM plays an important role in the regulation of lung cell proliferation and differentiation during repair of the bronchiolar airway epithelium.

### **A.3 EARLY TOXIC EFFECTS OF NIGRIN B ON MOUSE INTESTINE MUCOSA**

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The lectin nigrin b belongs to type 2 ribosome-inactivating proteins (RIL) that participate in plant defense against insects and pathogens. Because of it displays ribosome-inactivating activity it has been used in drug targeting for anti-cancer immunotoxins and as conjugate preparation with anti-tumor activity. The aim of the present study was to ascertain early histological changes in the small intestine after a lethal intravenous dose of nigrin b.

We have extracted nigrin b from elderberry bark and highly purified it by affinity chromatography. A lethal dose of 16 mg/Kg body weight was administered to 14 female Swiss mice, while 4 animals served as controls. Half of the animals were sacrificed at 6 h and the other half at 16 h after injection by intracardiac perfusion with 1% paraformaldehyde plus 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Samples of duodenum were taken and embedded either in paraffin wax or Spurr for light and electron microscopy, respectively. Care and manipulation of the animals followed the guidelines of the European Communities Council (2010/63/EU).

As early as 6 h after administration, the general structure of the small intestine is preserved. The villi appeared well structured showing hyper-cellularity in their core. Crypts appeared swelled with a large number of cells showing signs of apoptosis. 16 h after administration of nigrin b, cell destruction was more severe, villi appearing atrophic, as masses of loose connective tissue surrounded by intestinal epithelium devoid of goblet cells. Crypts have lost most of their cells. Paneth cells appeared a little more resistant, although finally they also undergo apoptosis.

Electron microscope analysis revealed swelling of the rough and smooth endoplasmic reticulum, cell membrane disruption and signs of apoptosis. Paneth cells showed a disorganized rough endoplasmic reticulum. Enterocyte microvilli were preserved as well as their glycocalyx and characteristic junctional complexes. Paneth cells also showed breaks in their nuclear envelope and chromatin condensation.

In contrast to lethal doses, sub-lethal concentration of nigrin b produce reversible effects, what means that the intestinal stem cell niche is not totally eliminated, thus allowing regeneration. Therefore, treatment of intestinal tumors bearing mice with nigrin b would prove helpful to study the growth of these tumors. Since the concentration of nigrin b required to inhibit translation is higher than that to affect cell viability, and according to our results, the effects of nigrin b seem to be pleiotropic and probably comprise translation arrest along with the promotion of other different mechanisms triggering apoptosis, such as unfolded protein response or releasing of macrophage-derived cytokines, as seen in another RIL like ricin.

Intravenous administration of a lethal dose of nigrin b exerts specific toxic effects in the small intestine within 6 h, promoting apoptosis of all cell types in the crypts. Sub-lethal doses of this toxic could be potentially useful to trigger the destruction of transformed cells present in the crypts leading to cancer.

Funded by Junta de Castilla y León (GR106).

#### **A.4 STUDY OF THE AUDITORY THRESHOLD AND THE COCHLEAR NUCLEI INTEGRITY IN A PERINATAL ASPHYXIA MODEL**

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Audition defects caused by hypoxic-ischemic encephalopathy give rise to language retardation with very important repercussions in daily life. To evaluate these auditory capacity in newborns, it is recommendable to use quantitative measures methods such as auditory evoked potentials (AEPs) that reflect the voltage variation produced during the auditory pathway after a short acoustic stimuli.

The objective of the present work was to determinate the functional integrity of the auditory pathway by the measure of the AEPs and to visualize the damage with histological methods in the cochlear nuclei using a model of hypoxic-ischemic brain injury in neonatal rats.

7-day-Sprague-Dawley rats were randomly assigned to two experimental groups: SHAM operated animals (pups with neither ischemic nor hypoxic injury) and hypoxic-ischemic (HI) group, animals submitted to both ischemia (permanent left carotid occlusion) and hypoxia (reduction of O<sub>2</sub> to 8%) by Rice-Vannucci method. The AEPs were measured by the GSI Audera equipment at 40 days and then, animals were sacrificed and the cochlear nuclei isolated, paraformaldehyde fixed, paraffin included, sectioned and stained with hematoxylin-eosin.

The auditory threshold for all these animals was 35Db, the highest frequency with no response to the audition stimuli. In the study of the AEP, the HI group showed a smaller amplitude signal after the stimulus in comparison with the control group. This result was more evident in response to the 75Db stimuli.

At histological level, the cells from the cochlear nuclei, of the SHAM group showed the normal morphology with a differentiated nucleus and nucleolus and visible neuronal prolongations. However, in the HI group the cells were more stained, with condensate cytoplasm and also surrounded by a tissue retraction zone.

Our results suggest that the HI event reduces the auditory capacity and generate neuronal damage in the cochlear nuclei, being these results an interest start point for the development of future assays in order to reduce neonatal hypoxic-ischemic induced hypoacusia.

**Acknowledgments:** This work was supported by grants from the Basque Country Government (IT773/13) and Fundación Jesus de Gangoiti Barrera.

## **A.5 STUDY OF GASTRIC HISTOPATHOLOGY FOLLOWING SLEEVE GASTRECTOMY IN A MODEL OF DIET-INDUCED OBESE RATS**

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Obesity prevalence is reaching epidemic proportions in the current society. Bariatric surgery represents an effective treatment for carefully selected morbidly obese patients, leading to long-term weight loss and a substantial improvement of obesity related co-morbid conditions. Sleeve gastrectomy (SG) has become an effective bariatric surgical technique. Follow-up data have demonstrated that SG provides important weight loss and resolution of comorbidities even 3–5 years post-operatively. Nonetheless, SG presents some disadvantages and associated risks derived from the direct intervention on the stomach. The aim of the present study was to analyze, in a model of SG in diet-induced obese rats, the histopathological evolution of the residual stomach at different times (4, 16 and 18 weeks) after the surgical intervention. In all the sleeve-gastrectomized rats we have found traits of gastritis cystica profunda, which became more severe with time. This lesion is characterized by gastric foveolae elongation with hyperplasia and cystic dilation of the glands in the sutured gastric area. After 18 weeks of undergoing the SG, foci of pancreatic acinar and ciliated metaplasia signs were observed occasionally. All the lesions were localized in the area close to the suture line, with no extension towards the rest of the gastric mucosa and were usually surrounded by abundant eosinophilic inflammatory infiltrate, numerous macrophages and mast cells. The microscopic changes observed could be related with a cytoprotective mechanism against gastric suturing. Although rat and human gastric anatomy differ considerably, this information could be of great importance, especially in patients with complications following SG.

## A.6 MORPHOMETRIC STUDY OF MICROVASCULAR DENSITY IN PANCREATIC DUCTAL ADENOCARCINOMA.

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a high mortality rate. Angiogenesis is regarded as an essential factor for tumor growth but this process remains controversial in PDAC. The aim of this work is to study the microvascular density (MVD) in a series of 60 pancreatic tumours and compare it with the tumoral grade and the clinical tracing.

We study the main clinical and pathological features of sixty PDAC and we correlated them with the microvascular density (MDV). Hematoxylin & Eosin staining was used to visualize the tumor center and margins and also to note variations in the degree of differentiation. Tumor histology was classified according to the 2000 WHO criteria. Avidin-biotin technique with anti-CD31 immunohistochemical staining was also performed for all cases for quantify la microdensidad vascular.

We found that moderately differentiated pancreatic adenocarcinoma predominated, while poorly differentiated adenocarcinoma was identified in only 8 cases. The total number of vessels, the number of capillaries and number of arterioles were higher in tumor samples than in peritumoral and normal samples (p value between 0.000 and 0.003 using ANOVA and Student's t-test). A positive association was found between total number of vessels and tumor differentiation (p<0.05). The total number of vessels was higher in the well and moderately differentiated carcinomas (p value 0.0015). No significant relationship was found between vascular parameters and other clinicopathological characteristics including age, sex, tumor location, tumor grade, vascular and perineural invasion, lymph node metastasis and distant metastasis.

We concluded that the microvascular density is higher in pancreatic ductal adenocarcinoma and that the number of microvessels is higher in well and moderately differentiated tumours, as opposed to the poorly differentiated pancreatic adenocarcinoma.

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## A.7 EVALUATION OF THE QUALITY OF DIGESTIVE ENDOSCOPIC BIOPSIES.

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The absence of appropriate biopsy sampling can cause a significant reduction in the quality of tissues, reducing the utility of such specimens.

Previous studies have compared several biopsy forceps currently available for use with varying diameters to determine whether design influences tissue adequacy in sampling for digestive diseases. There are limited data as to which forceps size is superior for obtaining optimal tissue samples. They have often used the diameter of the biopsy specimen as a surrogate for quality of sampling. However, proper interpretation of biopsy specimens by a pathologist requires that the sample also be properly oriented and at least to the depth of the muscularis mucosa. We would propose creating a standardized scoring method of biopsy material. There are no scientific references about such an intervention for histopathologic purposes.

Evaluation of the quality of digestive tract biopsies to assess the clinical-pathological diagnostic correlation and the overall adequacy of specimens for histological diagnosis providing suitable indicators of the quality of endoscopic procedure, and clinical suitability.

This is a retrospective, single-center, randomized study in relation to digestive endoscopy. A total number of 2906 biopsy specimens carried out between 2006 and 2010 in the laboratory of Pathology of La Línea Hospital (Cádiz, Spain), underwent a technical quality score system to assess the adequacy of the tissue samples of biopsy for the anatomopathological diagnosis. Also, the clinical-pathological diagnostic correlation was performed. Statistical analysis was assessed ( $p < 0.05$ ).

Only in 3 cases we could not perform the technical assessment but the diagnostic agreement.

The primary outcome, adequacy for pathologic assessment, was defined a priori. Specimen adequacy was defined a priori as a well-oriented biopsy sample 2 mm or greater in diameter, minimal crush artifact ( $< 50\%$ ), and with at least muscularis mucosa present. A relatively high proportion of biopsy specimens were well oriented ( $65.3\%$ ,  $P < 0.05$ ), resulting in a high overall adequacy rate ( $63.3\%$ ,  $P < 0.05$ ) and a low rate of biopsy samples deemed inadequate ( $36.7\%$ ,  $P < 0.05$ ). With regard to the presence of muscularis mucosa, there were  $65.0\%$  ( $P < 0.05$ ) of specimens with enough depth.

Histologic depth and orientation are significantly major determinants of the diagnostic adequacy of the specimens. Other standards deemed relevant are crush artefact and location. However, as demonstrated by the current study, larger specimens do not necessarily always translate into superior tissue quality.

The clinical-pathological diagnostic correlation reached a percentage of  $85.5\%$ . Location is its major determinant.

Few data exist with regard to biopsy quality for histologic assessment. The study demonstrated that the biopsy parameters of our quality score system are digestive tract endoscopy quality indicators, and likely a part of a continuous improvement initiative of the endoscopy quality.

Some  $36.7\%$  of specimens considered histologically inadequate in this study by not achieving the quality standards, had been previously diagnosed.

## **A.8 EGFR-TARGETED NANOCARRIERS FOR DRUG DELIVERY IN THYROID CANCER TREATMENT**

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Nanotechnology has led to the development of drug delivery vehicles and diagnostic devices for the detection and treatment of several diseases, by using nanocarriers (NCs) which transport the therapeutic agent specifically to the pathological area. Among many possible applications, tumors have been the most often investigated. Thyroid cancer is the most common endocrine neoplasia, and although differentiated cancers have in general a very good outcome, undifferentiated tumors, as in the case of anaplastic thyroid cancer, are more aggressive and do not respond to treatment. It is known that NCs accumulate in the pathological area by the enhanced permeability and retention (EPR) effect of macromolecular particles, which is a universal phenomenon of solid tumors related to their anatomical and pathophysiological differences from normal tissues. Besides the fact that NCs are accumulated, we propose to target anticancer drugs to epidermal growth factor receptor (EGFR), a molecule known to be overexpressed in a number of cancers. In order to develop a targeted cancer therapy strategy, not only we need to test the ability of NCs to stimulate internalization but also their intracellular transport and fate, since trafficking to lysosomes would lead to drug degradation.

To evaluate this strategy we coupled an antibody against the epidermal growth factor receptor (anti-EGFR) to the surface of 100 nm FITC-labeled prototype polystyrene nanocarriers (anti-EGFR NCs) and tested their binding, endocytosis, and lysosomal transport by fluorescence microscopy in human thyroid follicular cells, either control (Nthy-ori 3-1), cells derived from a follicular carcinoma (FTC-133), or an anaplastic thyroid cancer cell line (8505C).

Our results show that anti-EGFR NCs bound to all tested cell types with efficiency and specificity, validating the adequacy of this strategy for targeting thyroid follicular cells. Targeting was similar for cancer thyroid cells FTC-133 and 8505C (2772±694 and 3661±368 NCs per cell, respectively), whereas it was lower for control cells (418±73 NCs per cell). Furthermore, although the three thyroid cells lines internalized approximately 40% anti-EGFR NCs within 1 hour, NCs transport to Texas red dextran-labeled lysosomes was delayed in 8505C cells compared to control Nthy-ori 3-1 cells. Our results provide an avenue to explore anti-EGFR NCs as transporters to specifically deliver therapeutic agents to thyroid cancer cells.

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### A.9 PATIENTS WITH LIVER DISEASE PRESENT ALTERATIONS IN ORAL CYTOLOGY: INVOLVEMENT OF MINIMAL HEPATIC ENCEPHALOPATHY

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Patients with liver cirrhosis may present minimal hepatic encephalopathy (MHE) that can be unveiled using specific neuropsychologic examination. Early detection of MHE would be very useful. The “gold standard” for MHE diagnosis is the psychometric hepatic encephalopathy score (PHES). However, it is time consuming and needs adjusting for age and educational level. It would be very useful to have some biomarker reflecting the presence of MHE in cirrhotic patients. It is known that high percentage of cirrhotic patients have diabetes mellitus type II. The aim of this work was to study whether changes in oral cytology could be used as biomarkers for MHE, since both liver cirrhosis and diabetes mellitus type II lead to oral cytological alterations.

We analyzed lingual cytology from 33 controls, 42 cirrhotic patients without MHE and 14 patients with MHE. Patients were subdivided in four groups according to psychometric tests and the presence of diabetes: NMHE (patients without MHE nor diabetes); MHE (patients with MHE without diabetes); NMHE+D (patients without MHE with diabetes), MHE+D (patients with MHE and diabetes).

The samples were obtained by scraping the lateral portion of the tongue and stained by Papanicolaou's technique. Images were analyzed by Image Pro Plus: eosinophilic cells (Eo), basophilic cells (Bas), amphophilic cells (An), cornified cells (Cn), basal cells (Bs), pyknotic nuclei (Pic) and vesicular nuclei (Vn).

Expressed as percentages:

	n	Eo	Bas	An	Cn	Bs	Pic	Vn
CONTROL	33	58±3	23±2	12±2	6±1	0.4±0.1	71±3	29±3
NMHE	30	51±3	25±3	13±1	6.6±1.4	0.6±0.1	62±3	41±3
MHE	7	41±7	34±10	6±1*	8.6±5	1.4±0.9	45±10*	10±2***
NMHE+D	12	53±7	39±4	12±1	3.0±0.7	0.6±0.2	69±5	33±5
MHE+D	7	23±3 <sup>a,*</sup>	51±8 <sup>b,**</sup>	5.7±2*	9±4*	0.7±0.4	46±7*	26±2

Values are expressed as mean ± SEM. Significant differences between patients and controls are indicated as a p<0.05, bp<0.01. n: number of subjects. Differences between NMHE and MHE or NMHE+D and MHE+D patients are indicated as \*p<0.05, \*\*p<0.01, \*\*\*p<0.001

Statistically significant differences (p<0.05) between NMHE+D and MHE+D groups were observed for eosinophilic (p<0.05), basophilic (p<0.01), amphophilic (p<0.05), cornified (p<0.05) cells, and pyknotic nuclei (p<0.05). MHE patients without diabetes have also significant differences compared to NMHE group in amphophilic (p<0.05), pyknotic nuclei (p<0.05) and in vesicular nuclei (p<0.001). These alterations were greater in patients also having MHE and together with a progressive decrease of pyknosis, were indicative of a deficient epithelial maturation. Oral cytology alterations get worsen when diabetes mellitus type II is associated with cirrhotic process.

We have found good correlations between maturative alterations of oral cytology and MHE, and this could be a quicker way to anticipate the possible diagnosis of neurological deterioration, characteristic of cirrhotic patients with minimal hepatic encephalopathy.

### A.10 PATHOLOGICAL FINDINGS IN NORMAL UNTREATED OLDER MICE

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Mouse pathology is becoming the gold standard for investigating the phenotype of mouse models of relevant human diseases. In this context, it is important to understand the morphological changes that occur during normal aging of mice, so we do not mistake them with genotype-related pathologies. In our laboratory there is a number of genetically modified mouse colonies which are studied as models of cancer, diabetes, and neurodegenerative diseases. During the years we have found numerous spontaneous pathologies. When an old animal with an unexpected pathology was found, it was sacrificed and tissue samples were fixed, processed, embedded in paraffin and sectioned. Tissue sections were first stained with a routine staining, hematoxylin-eosin, to study general tissue structure. In some cases, special stains were used, such as PAS, Masson's trichrome, and immunohistochemistry for specific antibodies. Here we offer a summary of the most common. These include osteoid osteomas, lymphomas, polycystic kidneys, malignant tumors, choroidal melanomas, and rectal prolapses. These results clearly show that care must be taken to identify pathologies that are related with normal degeneration of older mice in order to not mistake them with genotype-specific changes in genetically altered mice.

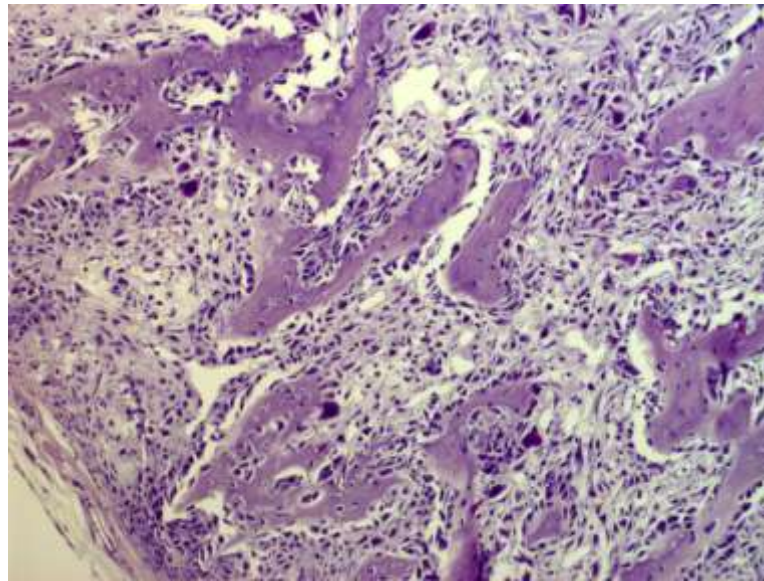


Figure1. Tissue section of an osteoid osteoma stained with hematoxylin-eosin (10x). This pathology was found in an 84 week-old C57B6 female mouse

## **A.11 LACK OF ADRENOMEDULLIN IN MOUSE ENDOTHELIAL CELLS RESULTS IN DEFECTIVE ANGIOGENESIS AND VASCULAR PERMEABILITY *IN VIVO***

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Adrenomedullin (AM) is a vasodilating peptide involved in the regulation of circulatory homeostasis and in the pathophysiology of certain cardiovascular diseases. AM is constitutively secreted by vascular endothelial and smooth muscle cells and is expressed at a high level in the lung and tumor cells. AM plays critical roles in blood vessels, including regulation of vascular stability and permeability. Besides, AM is also essential for angiogenesis. Previous studies showed that mice lacking AM develop cardiovascular abnormalities, including overdeveloped ventricular trabeculae and underdeveloped arterial walls. So that embryos lacking AM signaling die due to leaky and unstable blood vessels. Therefore, AM is indispensable for the development and/or maintenance of the vasculature during embryogenesis.

Conditional knockout of AM in the endothelial cells (AM<sup>EC</sup>KO) was obtained by crossing animals whose *adm* gene was flanked by *LoxP* sequences with transgenic mice expressing Cre recombinase under the *Cdh5* promoter.

In the liver of AM<sup>EC</sup>KO animals, large accumulations of small mononucleated cells (mostly lymphocytes) were found in the proximity of large blood vessels. In the kidneys, excessive deposits of Sirius red-positive material (collagen I) were found in the Bowman's capsule of the glomeruli in AM<sup>EC</sup>KO animals. In addition, mice lacking AM in their endothelial cells had defective angiogenesis *in vivo*. Besides, following injection with Evans blue dye, AM<sup>EC</sup>KO mice revealed increased vascular permeability in comparison with wild-type littermates.

Here we show that ablation of AM in endothelial cells caused the spontaneous development of liver immune infiltration and glomerulonephritis in mice. These findings revealed a physiological function of AM in protecting the liver and kidney from disorders such as inflammation, identifying AM as an immune suppressor in these organs. Moreover, the amount of vascularization in the matrigel implants was lower in AM<sup>EC</sup>KO mice indicating a defective angiogenesis. Furthermore, AM<sup>EC</sup>KO showed increased vascular permeability which suggests that AM plays an important role in the maintenance of vascular integrity.

Future studies will investigate the abrogation of the AM signaling pathway as antiangiogenic treatment of cancer in humans.

## **A.12 DIFFERENTIATION DELAY OF BRONCHIOLAR CLARA CELLS AND INCREASED POOLS OF LUNG STEM CELLS IN MUTANT MICE WITH CONDITIONAL DELETIONS OF IGF1R GENE IN THE LUNG EPITHELIUM**

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Insulin-like growth factors 1 (IGF1) and IGF2 can influence growth, differentiation, and survival of cells expressing their cognate type 1 receptor (IGF1R). IGF genes have been involved in both, normal lung development and lung repair after injury. They are also implicated in relevant respiratory diseases such as respiratory distress syndrome, idiopathic pulmonary fibrosis and lung cancer. IGF1R- and IGF1-deficient mice die at birth because their lungs are disproportionately hypoplastic and collapsed, with alterations in alveolar epithelium maturation and vasculogenesis. Furthermore, reduction in IGF1R signaling confers resistance to lung airway damage and protects against pulmonary fibrosis and oncogenesis. Despite its relevance, *in vivo* cell autonomous IGF1R function in the epithelial compartment of the lung is poorly known. To better understand IGF1R signaling in lung airway and alveolar type 2 epithelial cells, cell types that express the highest levels of IGF1R in the lung, we generated two conditional (*Cre/loxP*) lung epithelium-specific IGF1R knockout mouse models. By using a transgenic line that targets Cre recombinase to the airway Clara cells (*CCSP-Cre*) in *Igf1r floxed* mice with low efficiency, lungs of conditionally mutant animals developed normally with no evident abnormalities in size, morphology, histology or ventilatory function. An *Nkx2.1-Cre* second conditional mutant line, that drives Cre with better efficiency to the entire airway epithelium (bronchiolar and alveolar), showed higher deletion rates and decreased levels of *Igf1r* mRNA in the lung. This IGF1R deficiency generated histological alterations in the distal epithelium of terminal bronchioles, with thinning and lack of Clara cells, but apparently normal alveolar epithelial cells. During recovery after naphthalene-induced ablation of Clara cells, IGF1R deficiency increased the proliferation in epithelial cells of terminal bronchioles and a differentiation delay of that cell type. *Igf1r* deletion during epithelial cell differentiation increases the proportion of undifferentiated epithelial lung progenitor cells and generates bigger colonies of lung epithelial undifferentiated precursor cells when they were grown *in vitro*. These preliminary results suggest that lack of IGF1R in the lung epithelium of adult mouse causes a differentiation delay of epithelial Clara cells at the terminal bronchioles, and increased pools of lung stem cells. Preliminary results analyzing lungs of both mutant models will be shown and discussed.

### A.13 EXPRESSION OF CD34 AND ALPHA-SMOOTH MUSCLE ACTIN IN HUMAN BREAST CANCER

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Nm23 is a tumor suppressor gene inversely related to the metastatic invasion cell lines of breast carcinoma, demonstrating that high levels of this protein are associated with excellent survival probabilities. The aim of this study is to relate Nm23 with the tumor microenvironment through tumor stromal with marker smooth muscle actin ( $\alpha$ -SMA) and angiogenesis through human progenitor cell antigen (CD34), and determinate as the appearance of changes in the tumor microenvironment contributes to course of disease.

A total of 100 paraffin-embedded tissue blocks from patients with breast cancer were obtained from Hospital Madrid Montepíncipe, Madrid. Immunohistochemistry by ABC method was realized. Primary antibodies used were: Monoclonal antibody anti-human Nm23 (NOVUS BIOLOGICALS Littleton. USA), dilution 1:100; monoclonal antibody anti-human SMA (DAKO Carpinteria, California. USA), ready to use; monoclonal antibody anti-human CD34 class II (DAKO Carpinteria, California. USA). Quantification of the number of epithelial tumor cells for Nm23, quantification of vascular profiles for CD34 and immunopositive tracts in the stroma for SMA was performed using densitometric techniques with the Leica AF Metamorph 1.4. Statistical analysis was performed with the IBM SPSS Statistics 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp).

59 of the cases analyzed were positive for Nm23. Nm23 was expressed in cytoplasm of epithelial cells. Qualitative differences among samples were found without distinction between breast carcinoma type and degree. Significant high stromal SMA expression was detected in Nm23 negative cases ( $p < 0.05$ ). CD34 immunoexpression was observed in the endothelial cells and no significant increase was observed were Nm23 was negative. Microphotographs of immunoexpression of Nm23, SMA and CD34 are shown in figure 1.

We can therefore conclude that changes occur at the level of the epithelial cells and in the tumor microenvironment have influenced on the course of disease. So the importance of stromal interaction with epithelial cells is well established in tumorigenesis. For future clinical routine analyses, ideally a limited amount of treatment-predictive stromal and epithelial markers should be defined.

**Acknowledgments:** This study was supported in part by financial grant from San Pablo-CEU University and Santander Bank (USP-BS-PPC04/2011).

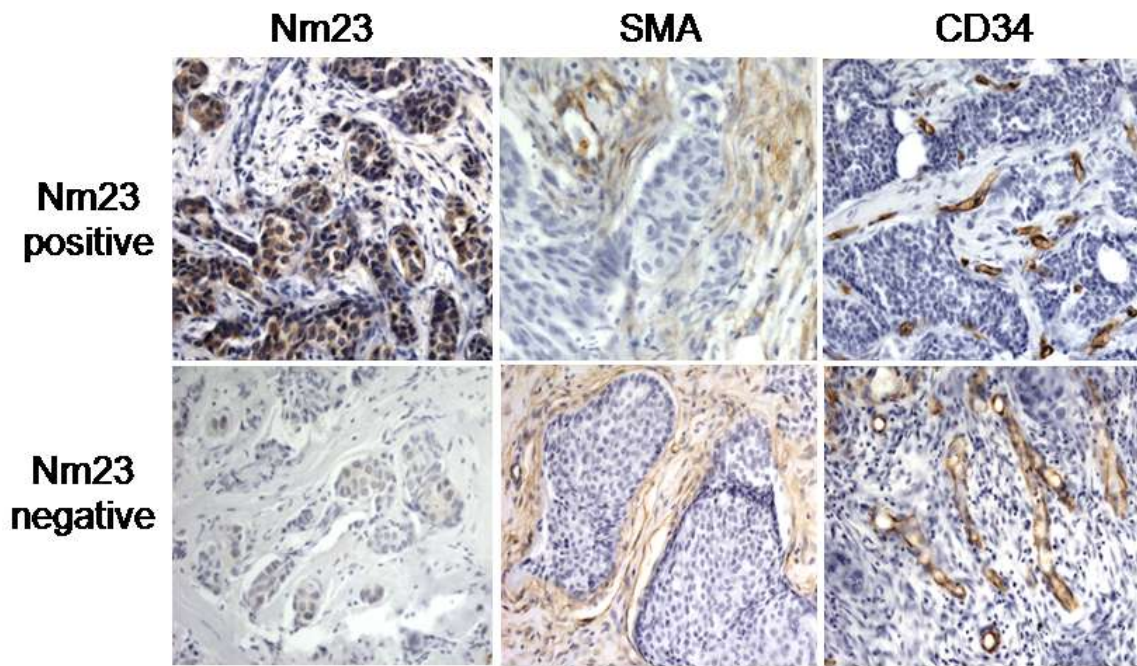


Figure 1. Nm23, SMA and CD34 expression in breast cancer. The upper line shows SMA and CD34 immunostaining when Nm23 was positive. The line below exhibit SMA and CD34 when Nm23 was negative.

#### **A.14 MONOSODIUM GLUTAMATE AS A PALLIATIVE AGENT AGAINST CISPLATIN-INDUCED ADVERSE EFFECTS**

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Monosodium glutamate (MSG) is a compound used as a food additive providing the taste “umami” in different foods. This taste might favour protein ingestion and could be used as a taste enhancer in special diets. On the other hand, in some animal models some of the complications typically associated to chemotherapy, such as anorexia, hyposalivation, alterations in gastrointestinal motility and peripheral neuropathy, have been reproduced and analysed. In addition to enhancing taste, MSG stimulates the glutamate sensors in the stomach and intestine, and activates exo and endocrine secretion and gastric motility. However, its effects on the alterations induced by antineoplastic drugs are unknown so far.

To evaluate the histological effects of the food additive monosodium glutamate in rats chronically-treated with the antitumoral drug cisplatin.

A quantitative study was performed by treating male Wistar rats (n=4 per experimental group) for 5 weeks with: A) cisplatin (2 mg/kg/week, i.p.); B) MSG in drinking water (4 g/l); C) cisplatin + MSG y D) water and saline (control group). Afterwards, samples from skin, small and large intestine, kidney and submaxilar salivary gland were fixed and processed for H&E or PAS staining, or for immunohistochemistry with anti-amylase (salivary gland) or anti-PGP 9.5 (peripheral nerve fibres) antibodies. A quantitative analysis was performed on salivary glands samples using the software Axiovision 4.6 followed by one-way ANOVA or t-student test.

MSG increased drinking water intake in control animals, without significantly modifying any of the histological parameters analysed. Previous data showed that chronic cisplatin reduces food intake and weight, alters gastrointestinal motility and induces painful peripheral neuropathy in the rat. Our present results showed that these functional alterations are not accompanied by alterations in the nerve fibres and Merkel cells in the skin, but histological damage of the gut wall was demonstrated, with colitis, ulcers and increased lymphocytic infiltration. In animals treated with cisplatin, MSG increased drinking water intake and partially improved damage to the gut wall. Additional histological observations were that in the salivary glands, the serous portion of the salivary glands was hypertrophied, whereas no significant change in immunohistochemical staining with alfa-amylase was apparent. In the kidneys, cisplatin induced tubular dilation and cell dysplasia without glomerular alteration, but MSG did not modify these effects.

MSG included in drinking water partially corrected damage to the small intestine and colon, suggesting that it could be useful as a palliative agent during chemotherapy.

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### **A.15 CLINICAL AND HISTOLOGICAL EVALUATION OF LIPOIC ACID TREATMENT ON KARWINSKIA HUMBOLDTIANA INTOXICATION IN THE WISTAR RAT.**

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The intake of genus *Karwinskia* plant's fruit causes flaccid, symmetrical and ascending paralysis which gradually progresses to either clinical recovery or towards death. Currently, the etiology is unknown and therefore, there is no specific treatment. It has been reported that one of the toxins isolated from the endocarp (T-514) increases production of free radicals *in vitro*.

To evaluate whether the clinical and histological manifestations present in this intoxication shows any improvement with lipoic acid, a potent known antioxidant.

We used a previously reported model of intoxication with *Karwinskia humboldtiana* fruit in the Wistar rat. In this model, a total dose of 3.5 g / kg of the ripe fruit was given, divided as follows: 1.5 g / kg on day 0, and 0.5 g /kg on days 3, 7, 10 and 14. We evaluated two schemes of lipoic acid administration in this model: the first group was administered with 30 mg / kg / day of lipoic acid orally for 15 days before intoxication, the second group was administered the same dose intraperitoneally for 44 days after intoxication. All animals were sacrificed at day 58, when paralysis was shown (as expected in this model of intoxication). We obtained and processed peripheral nerves, brain, cerebellum, spinal cord, lung, liver and kidneys to observe the histological preparations with light microscopy.

No clinical improvement was observed with any of the treatment schemes and no favorable changes were observed on the lesions in any of the organs studied.

Lipoic acid demonstrated no improvement on the clinical or histological lesions in the organs evaluated in this intoxication model. These results suggest that there may be other possible pathways that lead to histopathological damage in the studied organs, apart from the increase of free radicals that has been reported previously. In which that case, this intoxication would have to be studied in a more molecular approach to identify those mechanisms.

**KEYWORDS:** *Karwinskia humboldtiana*, lipoic acid, paralysis.

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### **A.16 TSH INCREASES "IN VITRO" PROLIFERATION RATE IN TT MEDULLARY THYROID CARCINOMA CELLS**

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Thyrotropin (TSH) is the main hormone involved in the regulation of thyroid function, controlling the synthesis and secretion of thyroid hormones (T3, T4) and, also, affecting the mitotic activity of follicular cells (FC). In recent years, our research group has demonstrated the expression of thyrotropin receptor (TSHR) in the other cell population of the thyroid gland, parafollicular cells, also known as C-cells (CC), in normal as well as in pathological CC of rat and human origins. In order to elucidate the role of TSH in the proliferation control of CC and its putative involvement in medullary thyroid carcinoma (MTC), we have investigated the expression of TSHR in human MTC tissue and the TSH-proliferative effects in the TT human-MTC cell-line.

RT-PCR and Western blot analyses were carried out to confirm the expression of TSHR in a human MTC cell line (TT). Moreover, immunohistochemistry on paraffin-embedded tissue sections from 15 MTCs was performed. To investigate the TSH effect in proliferation rates, cell proliferation was assessed by cytometry and variations in DNA synthesis were carried out by BrdU incorporation analysis. Finally, specific cell-cycle marker levels were measured by Western blot after TSH treatment.

TSHR strong signal was observed in all MTC tissue samples. TSHR expression was confirmed at mRNA and protein levels by RT-PCR and Western blot in the TT cell line. Differences in cell number and BrdU incorporation were observed in TSH-treated TT cells as well as in cyclin D1 protein levels.

Our results show a TSHR overexpression in MTCs and, in vitro changes in the proliferation rate and expression-increases in cell-cycle proteins after TSH treatment. These data provide a new avenue to be explored in molecular pathways regulating MTC tumor cell proliferation.

### **A.17 TISSUE EFFECTS OF BIOACTIVE PEPTIDES DERIVED FROM EGG PROTEINS ON OBESE ZUCKER RATS**

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Dietary habits play a key role in the development of the metabolic syndrome (MS). Functional foods, besides its nutritional properties, may produce beneficial effects on the organism and represent a favourable strategy for the prevention and treatment of this multifactorial disease. Bioactive peptides are a kind of functional foods, generated from food proteins by enzymatic hydrolysis both *in vivo* and *in vitro*, that have shown antihypertensive, antioxidant, hypocholesterolemic or antidiabetic activity, among others. However, there are very few studies that evaluate the effects of these enzymatic hydrolysates over tissues involved in metabolism.

To evaluate the effect of the consumption of egg protein extracts selected by their biologic multiactivity *in vitro*, on target tissues implicated in the metabolic syndrome related-endocrine-metabolic alterations.

Obese and lean Zucker rats were used. Animals were fed with standard diet since 8 to 20 weeks of life. Then, different experimental groups were performed depending on the drinking liquid: lean Zucker rats drinking water; obese Zucker rats drinking water; and other two groups of obese Zucker rats which received 0,75 g/kg/day of a peptide derived from egg white treated with pepsin (H1) or 0,75 g/kg/day of a peptide derived from egg white treated with aminopeptidase (H2). At the end of the different treatments, pancreas, liver, kidneys, epididimal fat and brown interscapular adipose tissue were fixed and dyed with H&E and Van Giesson. The image analysis was carried out with AxioVision 4.6 of Zeiss software.

The pancreatic islets in obese Zucker rats showed an irregular morphology and also a clear hypertrophy and hyperplasia in comparison with lean Zucker rats. This hypertrophy was similar in H1-treated obese Zucker rats but it was even higher in H2-treated obese Zucker rats. In the liver of obese Zucker rats, a clear steatosis was observed in comparison with lean Zucker rats. This steatosis is clearly diminished in H1-treated obese Zucker rats, but it is not modified in H2-treated obese animals. Renal damage was also observed in obese rats respect to lean animals, presenting dilated and very vacuolarized tubules. Neither H1 nor H2 provoked changes in this renal pattern. Adipocytes from the white fat of obese rats were clearly hypertrophic with respect to adipocytes from lean rats however, H1 consumption decreased cell size of these obese rats. Hypertrophy was even more evident in the brown fat of obese rats since their cells became, in fact, unilocular. No decrease in the size was observed after ingestion of H1 or H2.

The consumption of peptides obtained from the white egg treated with pepsin (H1), dramatically improves the degree of steatosis and the size of adipocytes in the white fat. It could be used for the prevention and treatment this type of alterations associated with the metabolic syndrome.

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### **A.18 MELATONIN INCREASES THYROGLOBULIN BIOSYNTHESIS IN THYROID FOLLICULAR CELL.**

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Melatonin is an indoleamine mainly implicated in circadian rhythm control of mammals and other vertebrates. Besides playing an important role as a transmitter of photoperiodic information, melatonin has antioxidant, anti-aging, antiproliferative, and, potentially, anticancerogenic activities, including suppressing indirect effects on secretory and growth processes of the thyroid gland. We recently demonstrated that melatonin is synthesized by C-cells and its receptors are present in follicular cells, thus suggesting a paracrine role for this neurohormone at thyroid level. In the present study, we have analyzed the effect of melatonin on the expression of the thyroid specific genes: thyroglobulin (TGB), thyroperoxidase (TPO) and natrium-iodide symporter (NIS) in the rat follicular PC-C13 cell line. We have also analyzed the expression level of two of the main specific thyroid transcription factors pax8 and ttf1.

Rat follicular PC-C13 cells were treated with melatonin at different times and doses. TGB, TPO, NIS, pax8 and ttf1 mRNA expression were studied by quantitative RT-PCR. TGB and pax8 protein expressions were analyzed by Western blot and immunofluorescence.

TGB expression was significantly higher at both mRNA and protein levels, whereas TPO mRNA expression decreased after melatonin treatment. No significant changes were observed in NIS mRNA. On the other hand, pax8 mRNA expression was significantly higher after treatment with melatonin, but no difference at protein level was observed. No changes in TTF1 expression were observed either at mRNA or protein levels.

In this study we have demonstrated that melatonin is involved in the regulation of thyroid specific-gene expression and its effect is more significant on TGB. These results are consistent with previous data and support the hypothesis of the paracrine role for C-cell-synthesised melatonin on follicular cells.

**A.19 EFFECT OF SEX AND DIABETIC STATE ON GLUCAGON-LIKE PEPTIDE-1 EXPRESSION IN GUT OF NON-OBESE DIABETIC MICE**

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Type 1 diabetes arises from an autoimmune process that selectively destroys pancreatic beta cells, developing an islet lymphoid infiltrate described as “insulitis”. The non-obese diabetic (NOD) is a murine strain that spontaneously develops a form of autoimmune diabetes similar to human type 1 diabetes, thus being the most widely used animal model for studying this disease. The development of diabetes occurs earlier in NOD females than in males, and affects a higher percentage of the population.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that is released from intestinal L-cells into the circulation in response to orally ingested nutrients, exerting a powerful insulinotropic effect in both normal and diabetic subjects. GLP-1 performs additional actions that suggest a therapeutic role in case of a deficit in  $\beta$ -cell mass, as GLP-1 increases it through stimulation of both  $\beta$ -cell proliferation and islet neogenesis and inhibition of  $\beta$ -cell apoptosis.

NOD mice (10 males and 10 females) aged 20 weeks were used. Glycemia and body weight were measured once a week. Pancreatic damage was evaluated by the degree of insulitis, endocrine area percentage and the mean number of islets. GLP-1 expression on the five gut regions (duodenum, jejunum, ileum, colon, rectum) was established by immunohistochemistry, and quantified by computerized image analysis.

Conclusions: 1) Females had a greater number of diabetic animals (30% vs 10%) 2) Insulitis in females was significantly higher than in males. Endocrine area percentage and the mean number of islets were significantly lower than in males. 3) GLP-1 cellular density was higher in the large intestine than in the small intestine. 4) Number of L cells increased significantly in all regions of males NOD mice. 5) There was no statistical difference in the density of GLP-1-immunoreactive cells between healthy NOD mice, prediabetic and diabetic.

## **A.20 OXIDATIVE THERAPY USING AMITRIPTYLINE IN COMBINATION WITH CAMPTOTHECIN IN HUMAN BREAST CANCER CELL LINES**

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Oxidative therapy is a promising anticancer strategy based on the production of high levels of ROS and/or the depletion of antioxidants of tumor cells. In this study we have focused on the potential antitumor activity of amitriptyline (AMIT), a tricyclic antidepressant, alone and in combination with the chemotherapeutic drug camptothecin (CPT) in human breast cancer cell lines.

Viability studies were performed by counting death cells with Tripan blue at increasing AMIT's concentrations. MCF-10A (non tumoral human cell line), MCF-7 (human breast cancer cell line, positive for estrogen receptors and negative for HER2) and MDA-MB-231 cells (human breast cancer cell lines, negative for both estrogen receptors and HER2) were treated 24h with AMIT (20 $\mu$ M), CPT (10 $\mu$ M) and a combination of both. Generation of ROS was studied using MitoSOX<sup>TM</sup>. Western blot of complex I and III were performed. ATP synthesis was determined in cell extracts using a luminometer.

MCF-7 cell line was more sensible to AMIT than MCF-10A and MDA-MB-231, being 20 $\mu$ M the lowest concentration at which the viability of MCF-7 was highly affected. Cell death was higher in the presence of AMIT in comparison with CPT, especially significant at 50 $\mu$ M. The combination of the drugs produced the highest damage. The level of ROS in breast tumor cells treated with AMIT was significantly increased respect to untreated cells in a dose dependent manner. This effect was potentiated with the combination treatment. Interestingly, basal ROS levels in non-tumoral cells were much lower than in breast cancer cells. Furthermore, complex I and III proteins decreased in both AMIT and CPT treatments, but the decrease was more accused when tumor cells were treated with the combination of the drugs. This effect was also observed in ATP production suggesting that AMIT induces mitochondrial dysfunction that ends in cell death.

Our results in breast tumor cells suggest that AMIT induces alterations in mitochondrial respiratory chain activity, increasing the generation of ROS until unviable levels. These effects were more pronounced in MCF-7 treated simultaneously with AMIT and CPT. AMIT is proposed to be tested, alone or in combination with different chemotherapeutic drugs, in antitumoral oxidative therapies in breast cancer cell lines and experimental tumors with molecular characteristics similar to those of MCF-7 cells.

**A.21 THE EFFECT OF *LOXOSCELES INTERMEDIA* VENOM IN MICE SKIN**

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In Brazil the spiders of genus *Loxosceles* are the major responsible by severe accidents. Their venom induces an intense dermonecrosis that is frequently irreversible. This effect had been studied only in rabbit. The phospholipase activity is one of the main responsible for development of lesion at the bite site in most of poisoning. Thus, this study aimed to establish an experimental model of lesion resulting from the local action of the *Loxosceles intermedia* venom in mice and test the phospholipase (PLA<sub>2</sub>) activity of the crude venom *in vitro*. All procedures were approved by the Ethics Committee for the Use of Animals of Instituto Vital Brazil. Different strains of mice were tested: Balb/C, C57/BL6 and Swiss. The animals were separated in two groups: control, which received saline injection and the group that received venom injection (341.4 µg of venom protein/100 µL). The saline solution and the venom were injected in the abdominal region by intradermal route (ID). A rabbit received the venom injection (ID) as positive control. Six and 24 hours after the venom injection, the animals were killed and the skin removed, processed and stained with hematoxylin-eosin. After 6 hours, no animals showed dermonecrosis at the inoculation site of venom. After 24 hours, all animals of strains Balb/C and Swiss died, however the C57BL/6 mice didn't showed local or systemic macroscopic effects. Six hours after the venom injection the skin of C57BL/6 exhibited an intense inflammatory infiltrate with scarce morphological change in the epidermis. On the other hand, the venom induced the dermonecrosis in the rabbit. The PLA<sub>2</sub> activity of the *L. intermedia* venom was measured by turbidimetric method with fosfatidilcoline as substratum. The absorbance at 925 nm was measured at initial time, after 15 and 30 min of reaction a 37°C. The *L. intermedia* venom not reduced the absorbance after 30 min, compared with negative control (substratum). The *Bothropos leucurus* venom was used as a positive control and reduced 50% of the absorbance at 15 min of reaction. These preliminary results suggest that C57BL/6 mice need more investigation about the inflammatory effects and more time of observation to aim this mice like a new experimental model to study dermonecrosis induced by *L. intermedia* poisoning. Moreover, *L. intermedia* venom has not PLA<sub>2</sub> activity, so more studies are necessary to investigate the venom activity responsible for dermonecrosis at the bite site.

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## **A.22 LOCALIZATION AND CHARACTERIZATION OF ENTERIC NEURAL PROGENITOR CELLS AND POSSIBLY MUSCULAR DIFFERENTIATION**

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Human myenteric plexus regeneration in the enteric nervous system is a proven fact, but it is still not known the process of regeneration of these nervous elements (neurons and glia). It has been recently reported that inside the ganglia, cells divisions only take place in glial cells. (Azan G et al 2011; Heanue TA et al 2010). It seems that some progenitors are also implied in smooth muscle regeneration. What are the cells that act as neural or muscular progenitors in this system?

In this work two hypotheses in relation to regeneration in the enteric nervous system are presented. Firstly, we suppose that progenitor cells are located inside the ganglia. These cells would come from glial cells. On the other hand it is suggested that stem cells would be located outside the ganglia. Other hypothesis is that these progenitors could be in relation to muscular cells differentiation.

Using proliferation cell markers (BrdU, Ki-67), we have found that dividing cells are located around the ganglia. To characterize these cells, we have proceeded to culture isolated gut cells from adult healthy mice and human tissue, with and without mucosa. In the present study the different cell morphologies have been evaluated and also the specific responses of these progenitor cells to specific antibodies. We have studied the expression of Nestin, GFAP, Neu N and actin protein in the isolated cells. FACS is suggested as the method to characterize enteric progenitor cells, using specific markers as c-Kit, p75, Nestin and GFAP. On the other hand, these cells were cultured with muscular differentiation media. Cellular morphology was observed during several days and also we studied the expression of a specific antibody for muscular cells (alpha actin).



### **A.23 CHOLECYSTOKININ-8 CELL DENSITY IN THE DUODENUM OF NON-OBESE DIABETIC MICE**

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Type 1 diabetes is a disease characterized by high blood glucose concentration, sometimes resulting in gastrointestinal symptoms. Gastrointestinal symptoms cause malnutrition in patients with diabetes, which, together with the disturbed immune defence in diabetes, may cause intercurrent infections. Gastrointestinal symptoms in patients with diabetes are attributed to disturbed gastrointestinal motility. The neuroendocrine system of the gut secretes peptides/amines that play an important role in regulating gastrointestinal motility. One of the gastrointestinal symptoms in diabetes is gastroparesis, which consists in delaying gastric emptying.

The non-obese diabetic (NOD) is a murine strain that spontaneously develops a form of diabetes similar to human type 1 diabetes, thus being the most widely used animal model for studying this disease. The gastric emptying of NOD mice is significantly slower than that of controls. The gastric emptying is inhibited by cholecystokinin-8 (CCK-8) secreted by I cells in the duodenum. Changes in its secretion may be related to gastrointestinal symptoms observed in diabetic patients, such a delayed gastric emptying.

The aim of this study was to investigate the changes of density I cells of duodenal mucosa in NOD mice. This study was performed in 10 males NOD mice aged 32 weeks, using NOD/Scid as controls. Glucose was measured once a week. The expression of CCK-8 in duodenum was established by immunohistochemistry, and quantified by computerized image analysis. Mice were separated into groups according to their glycemic status (normoglycemic, hyperglycemic and diabetic).

Conclusions: 1) Immunoreactive I cells are present in the epithelium of the villi and Lieberkhun crypts in the duodenum of NOD and NOD/Scid mice. 2) NOD mice have a higher density of I cells than the NOD/Scid but not significantly ( $p=0,476$ ). 3) Cell density tends to be higher in hyperglycemic NOD mice than in normoglycemic ones, but not significantly ( $p=0,115$ ).

#### **A.24 L-ENDOGLIN OVEREXPRESSION INCREASES RENAL FIBROSIS AFTER UNILATERAL URETERAL OBSTRUCTION.**

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Transforming growth factor  $\beta$  (TGF- $\beta$ ) plays a pivotal role in renal fibrosis. Endoglin, a 180 KDa membrane glycoprotein, is a TGF- $\beta$  co-receptor which is overexpressed in several models of chronic kidney disease but its function in renal fibrosis remains still unclear. Two membrane isoforms generated by alternative splicing have been described, L-Endoglin (long) and S-Endoglin (short) that differ from each other in their cytoplasmic tails, being L-Endoglin the most abundant isoform.

The unilateral ureteral obstruction (UUO) model of obstructive nephropathy reproduces the most representative features of tubulointerstitial fibrosis: extracellular matrix (ECM) accumulation, tubular apoptosis, myofibroblast activation and proliferation, tubular deletion and inflammatory cell infiltration, and it has been used in multiple studies to assess the mechanisms involved in these processes

The aim of the present study was to assess the effect of L-Endoglin overexpression in renal tubulo-interstitial fibrosis. For this purpose, a transgenic mouse which ubiquitously overexpresses human L-Endoglin (L-ENG+) was generated and unilateral ureteral obstruction (UUO) was performed in L-ENG+ mice and their wild type littermates.

Obstructed kidneys from L-ENG+ mice showed higher amounts of extracellular matrix proteins, such as type I collagen and fibronectin, than obstructed kidneys from WT mice. However, the higher increase of renal fibrosis observed in L-ENG+ mice is not due to a major abundance of myofibroblasts, as similar levels of  $\alpha$ -smooth muscle actin were observed in both mice types. Smad1 and Smad3 phosphorylation were significantly higher in obstructed kidneys from L-ENG+ than in WT mice.

Our results suggest that in vivo L-Endoglin overexpression potentiates Smad1 and Smad3 pathways and this effect is associated to higher renal fibrosis development

## **A.25 IMMUNOEXPRESSION OF LPA1, MCM7, EZRIN, AND PHOSPHO-EZRIN ON PRENEOPLASTIC CHANGES INDUCED BY CADMIUM CHLORIDE IN THE VENTRAL RAT PROSTATE**

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Cadmium chloride is an inorganic toxicant of great environmental and occupational incidence that might be implicated in human prostate carcinogenesis.

This study was directed to evaluate the immunoexpression of LPA1 (receptor of LPA), MCM7 (cell proliferation), Ezrin (membrane–cytoskeleton organizer), and Phospho-Ezrin in prostate of rats treated with cadmium chloride (Cd) and cadmium plus zinc chloride (Cd+Zn). Immunohistochemistry by ABC method was performed on ventral prostate lobe of control, cadmium chloride (Cd), and cadmium chloride plus zinc chloride (Cd+Zn) groups of Sprague Dawley rats of 24 months of age. The following variables were studied: percentage of cells immunoreactive to LPA1 ( $LI_{LPA1}$ ) and minichromosome maintenance protein 7 ( $LI_{MCM7}$ ), and percentage of immunopositive expression area of Ezrin and P-Ezrin.

The  $LI_{LPA1}$  and  $LI_{MCM7}$  were significantly increased in the animals treated in comparison with the controls. Percentage (%) of immunopositive P-Ezrin area was higher in Cd group while % of immunopositive Ezrin area did not show significant differences among the groups studied. The dysplasias (independently of their origin) were significantly increased for the  $LI_{LPA1}$ ,  $LI_{MCM7}$ , and % Ezrin immunoexpression in comparison with normal acini, and  $LI_{MCM7}$  and Ezrin immunoexpression showed statistical differences between treated-normal acini and dysplastic acini. No changes in P-Ezrin immunoexpression were observed. No significant differences between the two sources of dysplasias (Cd and Cd+Zn) respecting  $LI_{LPA1}$ ,  $LI_{MCM7}$ , % of immunopositive Ezrin and % P-Ezrin area were observed.

Our results suggest that LPA-1 and MCM7 might be implicated in dysplastic lesions induced by cadmium chloride development. Increase in percentage of immunopositive Ezrin and P-Ezrin area in dysplastic acini of Cd-treated animals can be suggested that epithelial cells of dysplastic lesions are becoming more invasive and gaining the ability to metastases to other tissues. Nevertheless, more studies are needed to confirm its potential contribution to the disease.

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## A.26 HISTOLOGICAL EFFECTS OF IONIC LIQUID [EM<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>OH][ESO<sub>4</sub>] IN EMBRYONIC DEVELOPMENT AND ADULT ZEBRAFISH

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Ionic liquids (ILs) are a class of molten salts with melting points below 100°C<sup>1</sup>. Typical ILs consist of an organic cation and a small inorganic anion, most often weakly coordinating halogen anions, such as chlorine (Cl<sup>-</sup>), tetrafluoroborate (BF<sub>4</sub><sup>-</sup>), hexafluorophosphate (PF<sub>6</sub><sup>-</sup>) or bromide (Br<sup>-</sup>)<sup>1</sup>. ILs possess new and interesting properties (negligible vapor pressure, high thermal stability, excellent solvation ability)<sup>2</sup>. Currently, they are seen as promising “green” substitutes for conventional organic solvents in manufacturing, processing and industrial applications. However, the toxicity of ILs both on organisms and the environment has also been questioned and some reports have revealed that the “greenness” of IL varies on depending of their chemical organization<sup>3</sup>.

The aim of this study was to determine the effect of the IL N-ethyl-N-(2-hydroxyethyl)-N-dimethylammonium ethylsulfate, [EM<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>OH] [ESO<sub>4</sub>], on gills and skin of adult zebrafish (*Danio rerio*), as well as to evaluate their effect on early embryonic development.

Adults zebrafish were divided in experimental groups which were exposed for 7 or 15 days to different concentrations of the IL (10 µg/L, 100 µg/L, 1 mg/L, 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L). One group of fishes was used as control. Samples of gills and skin were fixed in an aqueous Bouin's solution, embedded in paraffin and stained with hematoxylin-eosin, periodic acid-Schiff (PAS), alcian blue (AB, pH 2.5) and PAS-AB. During the embryonic period, we have used doses of the IL which were non-lethal in former essays with adult zebrafish (10 µg/L, 100 µg/L, 1 mg/L and 10 mg/L). Embryos were observed under a stereomicroscope each 24 hours during 5 days post-fertilization. Some embryos were histologically processed.

Results showed that in adult fishes goblet cells of gills and skin increase in those specimens exposed to non-lethal doses of ionic liquid. Furthermore, the primary lamellae of gills increase in thickness. These changes occur in a dose-dependent manner, but they are not affected by the time of exposition to the ionic liquid. During the embryonic development, the alterations observed were: inability to hatch, yolk sac alterations, skeletal deformities and swelling of the pericardial cavity.

These results indicate that: 1) in adults this chemical presents low toxicity at concentrations less than 25 mg/L; 2) in embryos doses of the IL tested, cause disturbs in hatching as well as in the cardiovascular system and skeletal deformities. Rate of mortality is not apparently increased in comparison with control embryos.

<sup>1</sup>Suarez et al. *J. Chim Phys*, 95 (1998).

<sup>2</sup>Gathergood et al. *Green Chem*, 6 (2004).

<sup>3</sup>Fatemi & Izadiyan. *Chemosphere*, 84 (2011).

## **A.27 HISTOMORPHOMETRIC CHANGES IN THORACIC AND ABDOMINAL AORTA SEGMENTS OF RAT RELATED TO AGING.**

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Histomorphometric study about histological structure and elastic fibers distribution in aorta of aging rats have not explored previously.

We studied elastic laminas and general histological structure in aging rats. We evaluated differences between thoracic and abdominal segments of aorta in relation to elastic fibers layers distribution, spatial organization and branching. These data of elastic fibers were also studied using confocal microscopy. Additionally, we have measured the distance between elastic fibers, as well as vascular wall thickness.

The study was carried out in male Wistar rats (n=10). All the animals were sacrificed at 22 months old. Thoracic and abdominal aortic segments were processed for histological procedures. The sections were stained with orcein, Masson's trichrom and picosirius red. Elastic autofluorescence were evaluated by confocal microscopy. Immunohistochemistry were used for detecting smooth muscle actin (SMA) and vimentin. Morphometric determination was processed by FIJI (ImageJ).

Elastic fibers in thoracic aorta segments have a more regular disposition (interlaminar distance  $16.7 \pm 5 \mu\text{m}$ ) than elastic fibers from abdominal aorta. Abdominal elastic fibers have an irregular pattern ( $34.1 \pm 9.1 \mu\text{m}$ ;  $p < 0.05$ ) and multiple branching areas, that have been confirmed by confocal microscopy. The layers of elastic fibers at thoracic aorta are 9, instead 6 that are presented in abdominal aorta ( $p < 0.05$ ).

Morphometric determination of the area occupied by elastic fibers referred to total tissue wall in thoracic aorta take up a higher proportion ( $0.27 \pm 0.04\%$ ) than in abdominal aorta ( $0.22 \pm 0.05\%$ ). Thoracic aorta thickness ( $131.9 \pm 16.9 \mu\text{m}$ ) is significantly higher than in abdominal aorta ( $115 \pm 23.5 \mu\text{m}$ ;  $p < 0.05$ ).

Immunohistochemical methods demonstrated SMA and vimentin expression in both thoracic and abdominal aortic segments. Positive SMA and vimentin cells showed higher population cells in abdominal aorta than in thoracic aorta ( $p < 0.05$ ).

Thoracic and abdominal aorta show different structure in aging rats. The thickness of thoracic aorta is significantly higher than abdominal aorta. Elastic fiber structure shows a higher disorganization at abdominal segment than in thoracic segment. Elastic fibers layers are higher at thoracic aorta. Significant higher populations of SMA and vimentin positive cells have been found in abdominal aorta in relationship with thoracic aorta.

## **A.28 HUMAN FEMORAL EPIPHYSIS AS A MODEL FOR ASSESSMENT OF PRIMARY STABILITY OF DENTAL IMPLANTS.**

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To correlate the area occupied by bone tissue with the values of the resonance frequency of dental implants placed in a human ex vivo model.

We studied a total of sixty dental implants of 4.5 x 13 mm (Astra Tech AB, Mölndal, Sweden) placed in excised human femoral heads for hip fractures due to osteoporosis (n = 7) or prosthetic placement as a treatment for severe osteoarthritis (n = 10). Primary stability was assessed by resonance frequency analysis (Osstell™ Mentor, Integration Diagnostics AB, Göteborg, Sweden) following the instructions of the manufacturer. The amount of bone was assessed on femoral biopsies taken with trephine at the time of implant placement by image analysis with ImageJ program on tissue sections stained with hematoxylin-eosin and Masson's trichromic.

The cortical bone on the femoral head was thinner in osteoporosis cases than in cases of osteoarthritis (460.7±213.5 vs. 750.1±300.6 micrometres). Scores obtained for implant stability (ISQ) showed high primary stability (>70) in 26 implants placed in coxarthrosis and only in 8 placed in osteoporotic bone. However, a small number of implants ISQ values were indicative of low primary stability (<60) (4/43 in coxarthrosis vs 2/17 in osteoporosis). Results show greater ISQ in osteoarthritis (73.52±1.92) than in osteoporosis (67.13±1.09), F(1,63)=15.229, p<0.001.

The ISQ determined by resonance frequency analysis is able to discriminate bone quality (osteoporosis vs. Coxarthrosis). However, there is no correlation between the bone and the area of primary implant stability.

### A.29 MORPHOLOGICAL ASSESSMENT OF ANTI TNF-A IN A MODEL OF RHEUMATOID ARTHRITIS IN DAB/1J MICE.

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Rheumatoid arthritis (RA) is an inflammatory disease of unknown etiology with a world-wide prevalence of approximately 1%. Tumor necrosis factor (TNF) antagonists are efficacious in reducing clinical and biologic markers of disease activity and in inhibiting the progression of structural damage in both early and late forms of RA.

To evaluate by histopathology score the effect of Etanercept® in an established mouse model of rheumatoid arthritis.

Rheumatoid arthritis was induced in 40 DAB/1J mice of 24 weeks of age by inoculating bovine collagen II plus Freund adjuvant. There were two groups: Control (n = 20) and treated with Etanercept® at doses of 2 mg every seven days (n = 20). The mice were sacrificed after 15 and 21 days. We evaluated macroscopic (clinical) and microscopic articular lesions of the four limbs. Articular, bone and cartilage injuries, the number of joints involved and the intensity of the inflammatory infiltrate were calculated semiquantitatively on a 4-point scale. The involvement of articular capsule (Pannus) and soft tissue were dichotomously assessed. Also an overall score for the sum of all variables was established.

After 15 days, the control group presents an intensity and joint involvement of moderate degree, with an average overall score of 6.85/12, which increases to moderate / severe (8/12) after 21 days. The group treated with Etanercept® presents moderate intensity (5.8/12) after 15 days, and maintained the same intensity (6/12) after 21 days.

There are no statistically significant differences between the control group and the treatment group after 15 days, but there are statistical differences in the biological variables after 21 days of treatment for all morphological variables assessed (p < 0.05, Student's t test).

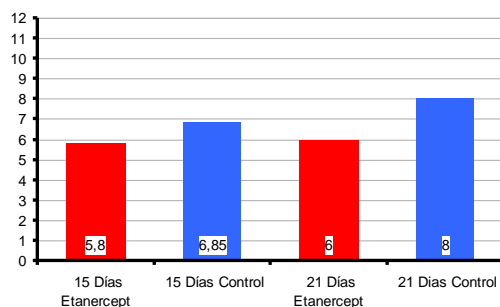


Table 1: Overall average in each group

Conclusion: Etanercept® improvement partially induced histopathological lesions in the murine model of rheumatoid arthritis.

### **A.30 IMMUNOHISTOCHEMICAL AND MORPHOLOGICAL STUDY OF CHRONIC PERIODONTITIS AND PERI-IMPLANTITIS. TWO ENTITIES WITH SIMILAR HISTOPATHOLOGICAL FEATURES.**

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Chronic periodontitis and peri-implantitis are two processes that share destructive inflammatory pathogenesis and morphological features. Discussions still take place today on whether they are or not two different clinicopathologic entities. Recent studies of transcriptomics seem to find a signature of different mRNA expression for both diseases.

To verify the diagnostic concordance rate between different observers for these two entities at morphological level and to study different leukocytes subsets present in both inflammatory pathologies.

We selected 10 random biopsies diagnosed with chronic periodontitis and 10 biopsies with peri-implantitis for a double-blind study with 3 independent observers. We assessed semiquantitatively the intensity of the inflammatory infiltrate in the lamina propria (score 0-3), and

we quantified in percentages the lymphocytes, plasma cells, monocytes / macrophages and polymorphonuclear cells by immunohistochemistry, as well as the presence or absence of bacterial colonies. Statistical analysis was performed with the kappa index using SPSS 20.0 program.

The Kappa index for diagnosis had a poor degree of agreement ( $k=0.2$ ). The kappa index between observers was good /very good ( $K>0.6$ ). The assessment of inflammatory infiltrate had a high degree of agreement ( $K=0.83$ ). The inflammatory infiltrate in the lamina propria was higher in the peri-implantitis cases ( $2.53 \pm 0.5$  vs.  $2.0 \pm 0.7$ ,  $p = 0.032$ ). These had a higher number of plasma cells ( $54.1 \pm 21.7\%$  vs.  $35.4 \pm 18.7$ ,  $p < 0.018$ ) and a similar presence of PMN, macrophages, lymphocytes and bacterial colonies ( $p > 0.05$ ). Leukocyte subsets show a similar proportion, not being statistically significant.

From the morphological point of view we do not observe differential features between chronic periodontitis and peri-implantitis, obtaining poor k indices of agreement inter observers.



## B. Tissue Engineering

### **B.1 TISSUE ENGINEERING: A NEW OPPORTUNITY FOR TRANSLATIONAL HISTOLOGY. THE HUMAN NERVE AS A MODEL**

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The recent development of novel methods allowing the efficient generation of artificial tissues by tissue engineering offers new perspectives and possibilities to medical histologists. Bioengineered tissues generated in the laboratory may be used to replace damaged tissues without the need of using allogeneic tissue grafts, but it is essential that these artificial tissues be biomimetic. While tissue engineering initially succeeded in building a number of human tissues, challenges still exist in developing complex tissue systems such as multifasciculated nerves using cells, biomaterials and bioactive signaling molecules. In addition, basic laboratory tissue models must fulfill several quality control criteria and be evaluated in laboratory animals before clinical translation from the bench to the bedside. In the present work, we describe the preclinical development of a human model of peripheral nerve substitute and the quality control process of these artificial tissues for future clinical translation.

First, we generated an artificial peripheral nerve model by tissue engineering using adipose tissue mesenchymal stem cells embedded in nanostructured fibrin-agarose biomaterials. Cells were isolated from small adipose tissue biopsies using enzymatic digestion with type-I collagenase. Once generated, the bioengineered nerve substitutes were analyzed *ex vivo* using biomechanical, histological and histochemical methods. Then, these artificial tissues were grafted *in vivo* in a laboratory rat model with a critical sciatic nerve defect, and the results were analyzed clinically, histologically and by electrophysiology.

The results of these analyses revealed that cells were able to grow and differentiate in the artificial nerves, without any sign of cytotoxicity induced by the biomaterial, and that these nerves had proper biomechanical behavior *ex vivo*. *In vivo* analyses showed that bioengineered nerves were highly biocompatible and properly integrated in the host nerve without any sign of malignant transformation. The use of these nerves was associated to a significant improvement of sensory and motor functions at the clinical and the electrophysiological levels. Histologically, a nerve regeneration process with axonal sprouting and remyelination was detected as compared to control animals. These promising preclinical results and the positive quality control process of the artificial human peripheral nerve will allow us to design a clinical trial in patients. For this purpose, the artificial nerve will be generated in a specific GMP (*good manufacturing practice*) facility and the required authorization of the Investigational Product (PEI) and the Manual for Researchers will be elaborated in accordance with the regulations of the Spanish Medicines Agency (AEMPS).

In conclusion, generation of biomimetic human tissues reproducing the histological pattern of native human tissues allows medical histologists to contribute to the clinical treatment of patients with tissue damage. Tissues for clinical use must be evaluated both at the *ex vivo* and *in vivo* levels before clinical translation in GMP facilities.

This work was supported by grant IPT-2011-0742-900000 from the Spanish Ministry of Science and Innovation (co-financed by FEDER).

## **B.2 HISTOPATHOLOGICAL ANALYSIS OF ARTICULAR CARTILAGE REGENERATION ON TISSUE ENGINEERED SCAFFOLDS**

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Physiologic cartilage repair leads to fibrocartilage filling of injury site, which lacks mechanical properties of hyaline cartilage, essential for weight bearing and load distribution. Cartilage tissue engineering by means of synthetic scaffolds can provide a mechanical setting favorable for hyaline cartilage repair.

The purpose of this research is to assess in vivo articular cartilage regeneration induced by tissue engineered scaffolds.

Scaffolds were synthesized by molding poly (ethyl acrylate-co-hydroxyethyl acrylate) copolymer with 90% of ethyl acrylate monomeric units, along with various cross-linking concentrations, obtaining 4 study groups with different stiffness. Biomaterials in form of discs were implanted in a 3-mm chondral defect on adult rabbit knees, after injuring subchondral bone to allow proper blood flow for cell repopulation purposes. Controls were submitted to similar layout, with no disc implantation. Animals were allowed to heal for 3 months. Regeneration was assessed through a modified cartilage repair score, morphometric and histological procedures.

Inserted scaffolds induced articular cartilage regeneration on injured surface as well as cell colonization of the implant. An inverse association of repair tissue with scaffold stiffness was observed, featuring increased fibrous tissue and less cell occupation in harder scaffolds. Overall developing cell population within the scaffolds was immature, not well differentiated, forming cartilage and bone clusters, together with scarce blood vessels and multinuclear cells. Controls originated articular cartilage regeneration with fibrous appearance when compared to native hyaline cartilage.

Tissue engineered scaffolds induced cartilage regeneration on the injured articular surface, holding an inverse correlation with the stiffness of the biomaterial. Furthermore, cell colonization of implant and integration with surrounding tissue was more fitting with softer biomaterials.

### **B.3 HYDROXYAPATITE BASED SCAFFOLDS WITH ELASTIN-LIKE POLYMERS FUNCTIONALIZED SURFACES IMPLANTED IN SUBCUTANEOUS AND BONE TISSUE.**

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The use of Elastin-Like Polymers (ELPs) for functionalizing surfaces is a novel advance that has been recently proposed. Among the bio-produced polymers, the ELPs are considered a family with a high potential for biomaterials design.

The aim of this work is to show the in vivo in soft (subcutaneous) and hard (bone) tissues performance of hydroxyapatite based scaffolds with elastin-like polymers functionalized surfaces. ELPs are designed with HA bond sequences and cell attachment specific sequences that interact with the inorganic phase and with the cells.

3-D periodic macroporous scaffolds were prepared via direct write assembly of a Si-HA slurry, using 3-D rapid prototyping printing equipment (envisionTEC 3D Bioplotter™). The slurry was formed by slow addition under stirring of 3 g of Si-HA Ca<sub>10</sub>(PO<sub>4</sub>)<sub>5.7</sub>(SiO<sub>4</sub>)<sub>0.3</sub>(OH)<sub>1.7</sub> powder over a 3 g polycaprolactone (PCL) solution on 20 ml of CH<sub>2</sub>Cl<sub>2</sub>. The final dimensions of the scaffolds were a mesh of 8 mm diameter and 2 mm height with interconnected cells, see (fig). Once the scaffolds were prepared the PCL employed during the processing stage was thermally removed at 600 °C for 6 h. These non-coated scaffolds are denoted HA-Si in the text below

Si-HA scaffolds were then coated by immersion with two different polymers: an elastin-like polymer designed specifically with hydroxyapatite binding domains (A recombinant amphiphilic tetrablock-copolymer with the aminoacid sequence MESLLP [{"(VPGVG)2-(VPGEG)-(VPGVG)2"}]10-[VGIPG]60}2V (abbreviated (E50I60)2) samples named HA-Si-ELR, and cell attachment specific sequences that will interact with the inorganic phase (HAp adhesion ones;SNA15 of the Stathenin) and with the cells (cell attachment domains; RGD loop of Fibronectin), samples named HA-Si-H4R4.

The 3 types of scaffolds were implanted in back subcutaneous and in a cavitory defect in rat femur. They were removed after two weeks and prepared for histological and immunohistochemistry studies.

Si-HA: after 2 weeks of subcutaneous implantation this biomaterial is observed encapsulated, with partial degradation and surrounded by macrophages which proceed to the degradation of the small nanoparticles the biomaterial is composed by. Cellular infiltration inside the scaffold with presence of osteoid-like cells is noticed. Si-HA, as scaffold in cavitory defect, presents a similar behaviour to subcutaneous implantation, with encapsulation and degradation of it and production of an osteogenic inductor stimulus around it, that contributes to the reparation of the defect from trabecular bone.

Ha-Si-ELR: in subcutaneous implantation, encapsulation is not uniform. Depending or not on the presence of elastomeric material, the scaffold shows areas with fibrotic cover besides areas where the material is surrounded by a macrophages like a garland. In cavitory implantation, neo-osteogenic stimulus is moderated inside the defect. The neo-osteogenic stimulus is more active thereupon the extremes of the defect, which allows the completely reparation of the wall defect.

H4R4: in subcutaneous implantation it is remarkable the great reactivity at the expense of monocytes/macrophages just like the presence of granulomas with intense inflammatory

reaction where biomaterial is degraded. In cavitory implantation neo-osteogenic stimulus inside the cavity allows partial reparation.

HA-Si and H4R4 prompt osteogenic stimulus from the medullar bone in cavitory implants. The coating with elastomeric peptide (ELR) modulate already said stimulus and accelerate the cortical restoration process.

This project was supported by Scaffide CIBER-BBN.

#### **B.4 HISTOLOGIC STUDY AND OSSTELL MEASUREMENT OF THE MACROSCOPIC DESIGN OF A NEW PROTOTYPE OF IMPLANT: MINIPIG EXPERIMENTAL MODEL.**

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The macroscopic design of a dental implant can stimulate different cellular specific answers according to its characteristics. It also has effect on dental implant primary stability and the ability to hold its charge during or after osseointegration. The correct design choice improves and keeps periimplant marginal bone avoiding the generation of high stress concentration at the implant neck area and consequent crestal bone resorption. The aim of the study is to determinate the causal relationship between the change of narrow essential cone 1'5 klockner implant core diameter and loops design and the biomechanic optimization of the bone to implant interface, by the determination of the bone-to-implant-contact (BIC) and the ISQ values measurements.

8 essential cone 1'5 klockner internal connection dental implants were placed in each right hemimaxilla and hind tibiae of 4 adult minipig females of 90kg weight and 10 months of age, switching different platforms (3'5 and 4 mm) of the standard model of this implant with a new prototype (core 0'2mm wider and more sharp turns). The animals were sacrificed 4, 15, 28 and 42 days after the implant insertion, measuring the osstell ISQ value at the time of sacrifice. Histologic study was performed by scanning electron microscopy (SEM). BIC was determined using the image analysis program Image J. The program Graphpad Prism 5 was used to perform the statistical analysis, comparing BIC results in hind tibiae (cortical bone) to maxillary (cancellous bone) in short (4 and 15 days) and long (28 and 42 days) term after implant insertion, in addition to the BIC percentages for the standard model and the new implant prototype.

No significant differences are evidenced between BIC, osstell and the macroscopic modification of the implant design, being the quality of the bone-to-implant interface better in the conventional implants. Significant differences between BIC percentages, at short term, of implants inserted in hind tibiae and those placed in the right hemimaxilla, were noted.

1) The conventional essential cone implant model of Klockner presents a greater BIC percentage than the new model. 2) There is a correlation between ISQ measurements and BIC. 3) The macroscopic changes of the new implant prototype did not involve a BIC increase at the long term.

## B.5 CHONDROGENIC INDUCTION OF ADIPOSE MESENCHYMAL STEM CELLS BY COMBINED ADENOVIRAL MEDIATED-GENE TRANSFER

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Articular cartilage has a poor regeneration capability; therefore numerous cell-based approaches to therapy are currently being explored. Adipose derived mesenchymal stem cells (ASCs) have the potential to differentiate into cartilage under stimulation with some reported growth and transcriptional factors and may constitute an alternative for cartilage replacement approaches. However, methods to effectively stimulate proliferation and subsequent chondrogenic differentiation of ASCs are needed. In this study, we analyzed the *in vitro* chondrogenesis of ASCs transduced with adenoviral vectors encoding the factors IGF-1, TGF- $\beta$ 1, FGF-2, and SOX9, either alone or in combinations.

Pellet cultures of characterized ovine ASCs were transduced with 100 MOIs of Ad.IGF-1, Ad.TGF- $\beta$ 1, Ad.FGF-2, and Ad.SOX9 alone or in combination, and harvested at various time points for detection of cartilage-specific genes expression by quantitative real time PCR (qRT-PCR) or after 14 and 28 days for histologic and biochemical analyses detecting proteoglycans, collagens (II, I, and X), and total sulfated glycosaminoglycans and collagen content, respectively.

Expression analyses by qRT-PCR showed that co-expression of IGF-1 and FGF-2 resulted in higher significant expression levels of aggrecan, biglycan, cartilage-matrix, proteoglycan, and collagen II (All with  $P = <0.001$  at 28 day). Aggregates co-transduced with Ad.IGF-1/Ad.FGF-2 showed selective expression of proteoglycans and collagen type II, with limited expression of collagens I and X, also demonstrated by histological analyses, and had significantly greater GAGs and collagen production than the positive control ( $P = <0.001$ ) and the other tested combinations. Western blot analyses also demonstrated increased expression of collagen II, while expression of collagens I and X were undetectable and limited for this combination, respectively.

ASCs co-stimulation with the factors IGF-1/FGF-2 enhances their chondrogenic differentiation by induction of chondrogenic markers, suggesting that this combination is more effective for cell-based therapies for cartilage repair than the other factors tested.

Keywords: Cartilage repair, adipose-derived mesenchymal stem cell, adenoviral vector, growth factors, chondrogenesis.

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## B.6 BIOINSPIRED ANTIMICROBIAL AND BIOCOMPATIBLE BIOCELLULOSE MEMBRANES AS TEMPLATES FOR TISSUE ENGINEERING

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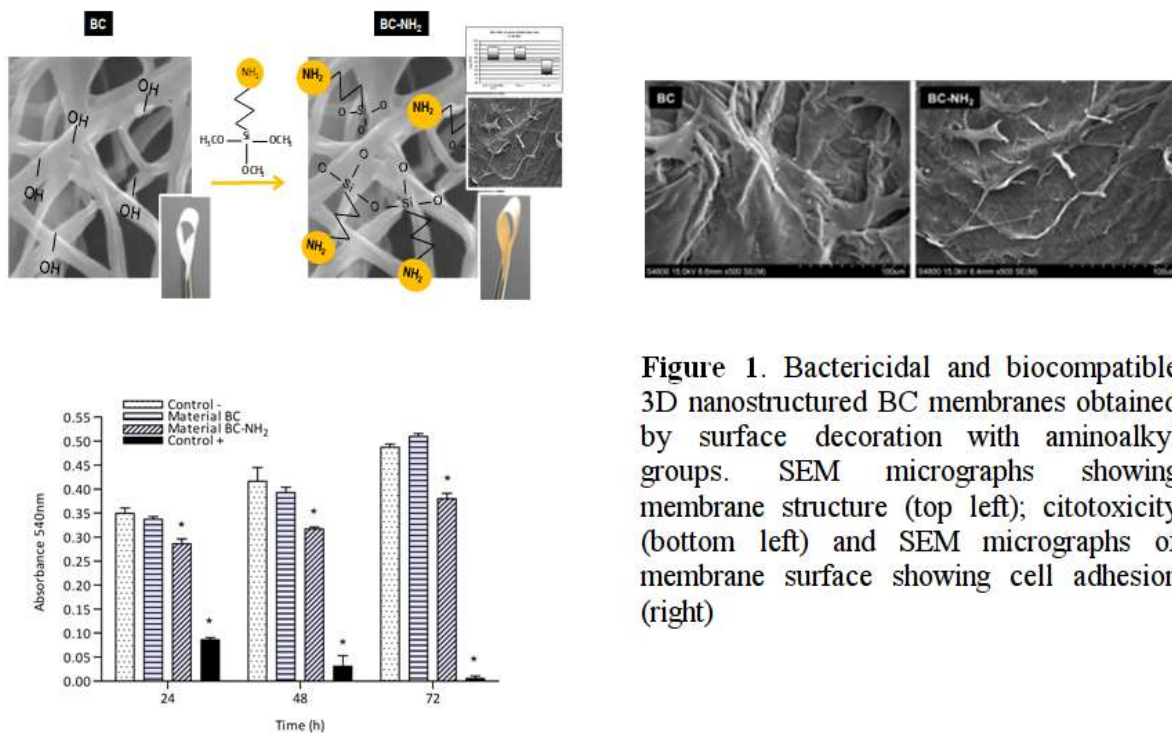
In recent years there has been a great interest in the use of 3D nanostructured biocellulose (BC) membranes for several biomedical applications, including tissue implants, wound healing and drug delivery. Recently, we have obtained antimicrobial BC membranes by the introduction of aminoalkyl groups into the surface of its nanofibrillar network, mimicking chitosan intrinsic antimicrobial properties. The aim of this study was the characterization of the aminoalkyl grafted BC membranes, firstly, in terms of cell response and, secondly, in terms of antimicrobial activity.

BC was produced by *G. xylinus* in the form of a swollen membrane (~99% water). BC membranes (BC-NH<sub>2</sub>) were obtained by chemical grafting of aminoalkyl groups onto BC, using aminopropyltrimethoxysilane. For evaluation of the cellular response to the grafted BC membranes, we used adipose derived stem cells (ADSCs), analyzing the cytotoxicity, proliferation and cell adhesion. The evaluation of antimicrobial activity was carried out by inoculating BC-NH<sub>2</sub> mats with Gram - (*E. coli*) and Gram + (*S. aureus*) bacteria.

Regarding *in vitro* cell response, as shown in Figure 1, standard growth values were obtained with the negative control (high-density polyethylene, HDPE) and a dramatic reduction of cell number was found with the positive control (polyvinyl chloride, PVC). In the case of cells cultured in presence of BC-NH<sub>2</sub> membranes extracts, a lower growth rate was found when compared with the negative control ( $p < 0.01$ ). However, considering that the viability and proliferation rates above 70% of the control were observed here, and that according to EN ISO 1093-5:200960 a material is considered cytotoxic if cell viability is reduced by more than 30%, we can state that BC-NH<sub>2</sub> mats are not cytotoxic for ADSCs.

SEM analysis was conducted in order to determine cell morphology, spreading and adhesion onto BC and BC-NH<sub>2</sub> membranes. As shown in the micrographs of ADSCs, taken after 72 h of seeding on the BC and BC-NH<sub>2</sub> membranes, cells were well spread, adhered correctly and proliferated to form a continuous layer of cells, fully covering the cellulose membranes. In the case of BC-NH<sub>2</sub> membranes, a lower degree of cell adhesion, spread and proliferation was observed compared to BC membranes. These results were in good agreement with the relatively lower growth rate obtained with cells cultured in extracted medium from BCNH<sub>2</sub>.

In relation to antimicrobial activity, for the culture containing the starting BC membranes, there was no reduction in bacterial cells; in fact, there was a 2-3log increase in growth of bacteria for *E. coli* and *S. aureus*. Nonetheless, BC-NH<sub>2</sub> membranes showed a significant reduction in bacterial viability for both *E. coli* and *S. aureus* bacterial strains after 24 h.



**Figure 1.** Bactericidal and biocompatible 3D nanostructured BC membranes obtained by surface decoration with aminoalkyl groups. SEM micrographs showing membrane structure (top left); cytotoxicity (bottom left) and SEM micrographs of membrane surface showing cell adhesion (right)

The introduction of aminoalkyl groups onto BC nanofibrils, using a silane chemical grafting approach, produces a BC membrane maintaining intrinsic biocompatibility whilst express antimicrobial activity. These novel BC-NH<sub>2</sub> membranes can therefore be seen as effective antimicrobial candidates in tissue engineering, tissue implants, wound healing therapy and drug delivery applications.



### **B.7 *IN VITRO* MESOTHELIAL CELL RESPONSE TO DIFFERENT REABSORBABLE PROSTHETIC MATERIALS DESIGNED TO REPAIR ABDOMINAL WALL DEFECTS.**

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Incisional hernia repair often requires the placement of prosthetic meshes in direct contact with the visceral peritoneum. This mesh-peritoneum interface could cause adhesion formation and further complications such as intestinal occlusion, abdominal pain or intestinal fistula. Structure and composition of the mesh are of great importance to ensure a proper mesothelization of the material surface. The aim of this study was to evaluate the *in vitro* response of peritoneal mesothelial cells in terms of their ability to cover different polymeric and biological meshes used in abdominal wall repair.

The following laminar absorbable prostheses were used: Bio-A<sup>®</sup> (BioA, Gore), Tutomesh<sup>®</sup> (Tuto, Tutogen Medical) and Strattice<sup>®</sup> (St, LifeCell Corporation). Meshes were cut in sterile conditions into 1 cm<sup>2</sup> fragments for subsequent cell seeding. No matrix or adhesion molecules that could enhance cell attachment were used.

Mesothelial cells were isolated from omental biopsies of White New Zealand rabbits (n=4). Tissue fragments were subjected to enzymatic digestion with 0.1% type-I collagenase and cultured in low glucose-DMEM medium containing 10% FBS and 1% antibiotics. Immunostaining of Cytokeratins 5, 18 and E-cadherin was developed to characterize the population. A density of 2-2.5x10<sup>5</sup> cells from the third passage was seeded in triplicate over each mesh fragment. The different follow-up times established were: 1, 4, 8, 16, and 24 h. At each time point, samples were fixed and processed for scanning electron microscopy evaluation. Surface covered by cells at the selected time points was measured, and percentage coverage of the different meshes was calculated.

Cultured mesothelial cells showed polygonal shape and formed a cobblestone-like monolayer when grown to confluence. Cells showed positive expression to Cytokeratin-5, E-cadherin, and mild presence of Cytokeratin-18.

The cells seeded on the polymeric BioA did not adhere to the mesh fibres at any of the time points. On the contrary, the cells seeded on the biological meshes Tuto and St were able to adhere to the surface, stretch and colonize different prosthetic areas. At 24 h post-seeding, St mesh showed small colonies of polygon-shaped cells with evident secretory activity to create a monolayer, and a percentage coverage of 12.03±0.91% was recorded. Tuto mesh showed dispersed cells that eventually formed small distant colonies that covered 8.74±1.36% of the surface 24 h after seeding.

At each of the time points, mesothelial cell cover for BioA was significantly lower than for both St (p<0.05 at 4 h; p<0.01, at 1, 8, 16 and 24 h) and Tuto meshes (p<0.01 at 4, 16, and 24 h; p<0.05 at 1 and 8 h). St coverage was lower than that of Tuto at 1 h (p<0.05) but became significantly higher at 16 and 24 h post-seeding (p<0.05).

Cultured mesothelial cells are not able to attach to the synthetic filaments of the polymeric BioA mesh;

on the contrary, collagen-based Tuto and St meshes allow these cells to colonize the biomaterial surface, promoting a better mesothelial coverage after 24 h of the seeding.

## **B.8 EXPERIMENTAL BIOASSAY OF A NEW ELASTIC NON-ABSORBABLE SUTURE (ASSUPLUS®) FOR MIDLINE LAPAROTOMY CLOSURE.**

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Closure of midline laparotomies is still a significant risk factor in the development of incisional hernias. Different types of sutures have been used in these procedures, failing to agree on what suture material to use. This study was designed to compare the biological behavior of a new suture with elastic properties, to that of conventional sutures (polypropylene and polydioxanone).

Three 2 cm midline laparotomies, 1 cm apart, were performed in 16 New Zealand White rabbits. The xifoid process was used as reference, beginning the laparotomies 3 cm away from it. The surgical wound was then repaired with an en masse running suture using three different 3/0 sutures: non-absorbable polypropylene (PP) (Surgipro®, Covidien); non-absorbable polyurethanic polyeter (PUe) (Assuplus®, Assut Europe) and absorbable polydioxanone (PDX) (Assufil®, Assut Europe). Two animals from each group were euthanized at 3 weeks and 6 months after surgery, and two animals not subjected to surgery were used as controls for each time point. Histological sections of the different specimens were stained with Masson's trichrome (Goldner-Gabe variant) and Sirius Red. Immunohistochemistry with RAM-11 antibody was performed to evaluate macrophage response.

Macroscopically, both at 3 weeks and 6 months, there was no evidence of wound dehiscence or complications. As expected, due to their non-absorbable nature, PP and PUe remained present at 3 weeks and 6 months after surgery. At 6 months, there were still traces of PDX.

At 3 weeks, all the sutures from the different study groups were surrounded by connective tissue composed mainly by collagen III, except for PUe, where the tissue had an increased presence of collagen I. At 6 months, the tissue was composed mainly by adipose tissue, with more collagen I than at 3 weeks, with no difference between any of the materials.

All the sutures presented a mild macrophage response at both time points. The non-absorbable sutures (PP and PUe) did not show significant differences between them. However, both sutures presented a significantly higher reaction than the absorbable suture (PDX) at 3 weeks ( $p < 0.01$ ). Such expression was significantly reduced for each suture between 3 weeks and 6 months ( $p < 0.001$ ). At 6 months, the macrophage response of PDX was slightly higher than that of the non-absorbable sutures, although this difference was only statistically significant when compared to PP ( $p < 0.05$ ).

The PUe suture presents efficient collagenization and a good foreign body response, equivalent to that of conventional PP;

The absorbable suture PDX shows a foreign body response which is more stable over time.

## **B.9 PERITONEAL SUBMESOTHELIAL TISSUE IS A POWERFUL SOURCE OF MESENCHYMAL-LIKE STEM CELLS IN ADULT MICE**

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Mesenchymal stem cells (MSCs) are widely used in tissue bioengineer and regenerative medicine. These cells have been detected and isolated from numerous tissues in adults. Here, we identified a new source of MSCs in the peritoneum of adult mice and proposed an innovative methodology to isolate, characterize and expand MSCs. Peritoneum is a thin two-layered membrane that covers most of the abdominal organs. Patients under peritoneal dialysis therapy usually develop peritoneal fibrosis and then ultrafiltration failure. MSCs are detected in peritoneal liquid and exudates, but the exact source is unclear. In this work, we investigated whether the anterior peritoneal wall of Balb/c adult mice is required to generate peritoneal MSCs. Fibroblastic-adherent moving cells from the explants were detected since 6 hours of culture until day 15, when the peritoneal wall fragment was removed from the culture system. Optical and electronic microscopy analysis of the fragments revealed submesothelial disorders, mainly where the mesothelium was devoid. Adherent cells remained proliferating in the culture system more 15 days (passage 0) and these cells were cytokeratin<sup>-</sup>, vimentin<sup>+</sup>, laminin<sup>+</sup> and a-actin<sup>+</sup>. At the end of third week, the adherent stromal cells were harvested with trypsin-EDTA and re-plated (weekly) until passage 3. Then, the phenotype was typically mesenchymal in approximately 95% of the cells (CD90<sup>+</sup>CD73<sup>+</sup>CD29<sup>+</sup>Flik-1<sup>+</sup>CD45<sup>-</sup>), these cells were also capable to differentiate into adipocyte- and osteoblast-like cells and expressed Sox-9. Moreover, these cells supported hematopoiesis for more than 4 weeks in a coculture system. Phase contrast analysis revealed dark (cobblestones) and bright (proliferative) cells strictly contacted with peritoneal stromal cells and stained cultured cells were well-characterized as myeloid progenitors. Corroborating, these peritoneal stromal cells expressed SCF, G-CSF, GM-CSF, IL-7, SDF-1 and TNF- $\alpha$ , suggesting the existence of potent genetic machinery to support hematopoiesis for long time, similar to bone marrow MSCs. In conclusion, we have proposed a new source of MSCs from submesothelial regions of the peritoneal cavity in adult mice and a distinct possibility to isolate, expand and application on tissue bioengineer and regenerative medicine.

## **B.10 ULTRASTRUCTURAL ANALYSIS OF MESENCHYMAL DIFFERENTIATION INTO CARTILAGE INDUCED BY PEA/PHEA SCAFFOLD**

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A scaffold biomaterial was developed to allow simple cell seeding and to analyze the samples reducing the further processing. The objective of the present work was to observe morphological changes in mesenchymal stem cells during proliferation and differentiation into cartilage.

The mesenchymal cells employed in the present work corresponded to the KUM5 cell line from murine bone marrow. For the construction of the scaffolds a PEA 90%/PHEA 10% copolymer was developed. Meanwhile, the cells were expanded in DMEM 4,5 g/L glucose with 10% FBS and 1% penicillin/streptomycin. After trypsinization and centrifugation, the samples were resuspended either in the same culture medium employed for expansion or in chondrogenic medium: DMEM 4.5 g/L glucose containing L-proline 50 µg/ml, ascorbic acid 50 µg/ml, dexamethasone 10<sup>-7</sup> M, ITS+Premix 1%, penicillin/streptomycin 1% and TGF-β1 10 ng/ml. Posteriorly, 25 µL containing around 50000 cells were seeded in each surface of the scaffold, and held for 40 minutes in standard culture conditions. Finally, the scaffolds were submerged either in expansion medium or in chondrogenic medium. The samples were retrieved for analysis at days 1 and 3 of culture. The fixation of the samples was performed in glutaraldehyde 2,5% for 1h at 4 °C, and histological analysis was carried out by means of optical semi-thin sections and transmission electronic microscopy (TEM).

The histological analysis performed on the samples cultured in expansion medium at day 1 revealed a large number of mesenchymal cells that were closely related to the biomaterial (Fig. 1). Meanwhile, in the samples cultured in chondrogenic medium, apart from the small, reticular mesenchymal cells associated to the biomaterial, a more differentiated population of bigger and rounder cells occupying the interstitial spaces of the scaffold was observed. When assessed with TEM, these cells expressed active-synthesis morphology, with bigger cytoplasm and dilated endoplasmic reticulum cisternae, but without evidence of any newly-generated extracellular matrix component. Both mesenchymal and synthesizing cells showed a marked tendency to cover the biomaterial through cytoplasmic expansions.

At day 3 of culture, the samples in expansion medium showed a smaller cell number with respect to the samples from day 1, appearing in a more dispersed and isolated pattern. On the other side, the samples cultured in chondrogenic medium showed a higher cell number and a higher tendency to differentiation. Additionally, voluminous round cells co-exist with small reticular mesenchymal cells, the first occupying preferably the interstitial spaces of the porous scaffold, and the second in close relation to the biomaterial surface. Evidence of a newly synthesized fibrillar material was identified with TEM both in the cytoplasm of those cells showing active-synthesis morphology and in the extracellular compartment.

## **B.11 ANALYSIS OF THE BONE REGENERATION CAPACITY OF ADULT STEM CELL FROM DIFFERENT ORIGIN**

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Bone graft therapy is applied in quality and quantity bone deficit because their osteogenic and osteo-inductive properties. Nevertheless, the morbidity in the donor zone and the long-term reabsorption mean an stimulus in order to perform a research of bone graft substitutes. The mesenchymal (MSC) stem cells are object of special attention due to its high plasticity. Originally isolated in bone marrow, can also be obtained from adipose tissue, umbilical cord or la dental pulp either adult or temporal. However, there are differences between them depending on the origin. This differences, even described in subpoblations of bone marrow, make of interest a detailed study of the osteogenic and osteo-inductive potentiality.

Extraction, characterization and comparasing of the sintetic activity from the different osteogenic factors of mesenchimal cells coming from different origin.

It was analyzed the intrinsic osteogenic activity of different human cells types: mesenchimal cells of adipose material, umbilical cord, pulpa dental of permanent and temporal teeth, commercial osteoblast, cells coming from bone microbiopsis, dental papilas and MG-63 line (osteoblast like cells from tumoral tissue). All cells were maintained in the same culture media (alfa-MEM+10%SBF) under standard conditions and they were analyzed in early stages (<6). It was used the kit of human bone metabolism Milliplex MAP (Millipore) which uses xMAP Luminex technology. This system allows the quantification of multiple metabolites based on flow citometry technology using poliestiren microballs attached to the specific antibody. In our case the osteocalcin, osteopontin and the osteoprotegerin as well as RANK-L present on the culture media. The samples were analyzed three times.

In relation to osteocalcin expression the highest results were found in cells coming from bone microbiopsia y adult dental pulp and the lowest in mesenchymals of umbilical cord, fat and temporal pulp. The osteoprotogerin was specially high in all dental pulp cells, fat, cord and bone microbiopsis, being less in the comercial osteoblasts. The osteopontin generated similar values in all cells except cord, papilla and adult pulp where values were higher. Lastly, the values obtained of RANK-L were found bellow the limit of the technical resolution in all cases.

Mesenchymal cells are far from having an homogeneous behavior. A priori there is no existence of a clear disposition of any mesenchymal cells to direct osteoblastic differentiation even though the differences in osteoprogeterin expression could condition its future behavior.

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**B.12 EVALUATION OF A MODEL OF BIOCOMPATIBLE ARTIFICIAL NERVE**

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Different biomaterials and technologies have been used to elaborate bioengineered conduits for nerve regeneration. These conduits should be natural, biodegradable, flexible, biocompatible and mechanically stable. Fibrin-agarose hydrogels are natural, biodegradable and highly biocompatible scaffolds, and their mechanical properties can be significantly enhanced by the process of nanostructuration. For these reasons, the objective of this work is to design and evaluate the physical properties of nanostructured bioartificial conduits made of fibrin-agarose hydrogels.

Acellular fibrin-agarose (FA) constructs were developed using human plasma and 0.1% agarose type VII (n=12). The FA constructs were nanostructured by plastic compression during 3 minutes using 300 grams, and conduits of 2.5 cm of length and 1.5 mm of diameter were developed. Native sciatic nerves from six male Wistar rats were used as positive controls (2.5 cm of length and 1,3-1,5 mm of diameter). Uniaxial tensile tests were performed using an electromechanical material testing machine (Instron, Model 3345).

The average values of Young's Modulus, tensile Stress at Break, and tensile Strain at Break were equal respectively to 0.317 MPa, 0.427 MPa, and 174.9% of deformation, while for the native nerves the values were equal respectively to 8.56 MPa, 2.14 MPa and 48.8% of deformation.

The FA bioengineered conduits at 0.1% of agarose showed good consistency and elasticity. The values of Young's Modulus and Stress at Break demonstrated that the native tissues are more rigid than the biomaterials, but the artificial nerves showed to be very elastic, as they are able to withstand three times more deformation before failure, than the native tissues.

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#### **B.14 TOPOGRAPHIC AND TIME-DEPENDENT EXPRESSION OF LAMININ IN TISSUE-ENGINEERED HUMAN CARTILAGE BASED ON FIBRIN-AGAROSE BIOMATERIAL**

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Cartilage represents a mesenchymal avascular tissue with limited repair capacity, thus suffering from a high prevalence of aging and degenerative-associated pathologies. Tissue-engineered cartilage constructs may represent a biologically and biomechanically ideal option of treatment. Laminin, is a ubiquitous component of basement membranes, and is related to tissue integrity and phenotype maintenance in some mesenchymal tissues. Nevertheless, the expression of laminin in cartilage is not homogeneous, varying in a timely and topographically manner. Also, some studies have linked the differentiation status of cultured chondrocytes with the three-dimensional structure of the scaffold in which they are cultured. The goal of our study was to generate human cartilage substitutes based on a fibrin-agarose scaffold, and to characterize, timely and topographically, the expression of laminin through immunohistochemical analysis.

Primary cell cultures of chondrocytes were obtained from human tissue biopsies. Then, fibrin-agarose stromal substitutes were generated with cultured fibroblasts immersed within, and the epithelial cells seeded on top. Hematoxylin and eosin staining, and immunohistochemical analysis of laminin were performed at 1, 3, 4, 5 and 6 weeks of culture.

Histological evaluation of the cartilage constructs revealed the proliferation of the chondrocytes in the fibrin-agarose matrix. Regarding the laminin expression, an intracellular pattern was observed since the first week of culture, with increasing intensity over time. The strongest staining was recorded at 6 weeks.

Our results suggest that the three-dimensional architecture of the fibrin-agarose biomaterial is adequate for promoting and maintaining the phenotypic characters of native chondrocytes.

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### **B.15 VALUATION OF EXTRACELLULAR MATRIX COMPONENTS IN ISOLATED HUMAN ATM FIBROCHONDROCYTES**

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Temporo-mandibular joint (TMJ) disc may be affected by numerous diseases and conditions. Autologous human fibrous chondrocytes implant could offer an alternative therapeutical approach for patient with TMJ disorders. However, chondrocyte cells kept in culture tend to de-differentiate upon long-term in in-vitro conditions, and the exact subculture in which these cells are appropriate for clinical use is not known. In this work, we have carried out a sequential study to evaluate the capability of cultured TMJ fibrochondrocytes to synthesize all major components of the fibrocartilage extracellular matrix (ECM).

In this work, we first isolated human temporo-mandibular joint fibrochondrocytes (TMJF) from human TMJ discs using enzymatic digestion with trypsin and collagenase, and cell cultures were maintained until the 9<sup>th</sup> subculture. Total RNA was extracted from cells corresponding to each cell passage, and Affymetrix oligonucleotide microarrays were used to determine the gene expression levels of the main genes encoding for ECM components along 9 consecutive cell passages.

Our results revealed that 32% of all genes encoding for fibrillar ECM components tended to reduce their expression along the 9 passages, especially after passages 5-6, including 2 genes encoding for collagen type I and procollagen (COL1A1, COL1A2, PLOD1 and PLOD2). The same was found for 17% of all genes encoding for glycosaminoglycans (chondroitin-sulfate, hyaluronic acid and heparan-sulfate genes), 24% of proteoglycan genes (aggrecan, biglycan and decorin) and 16% of all multiadhesive glycoproteins genes (nidogen, osteonectin and tenascin). In contrast, we found a significant increase of glycosaminoglycans genes (heparan-sulfate) and 10% of multiadhesive glycoproteins genes (LAMA4 and LAMB1).

These results suggest that TMJF tend to progressively lose capability of synthesizing fibrillar and non-fibrillar ECM components upon sequential culturing. The most appropriate ECM gene expression levels were found up to passages 5-6. For this reason, cultured TMJF should not be used in tissue engineering after passage 6.

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## **B.16 CELL VIABILITY QUALITY CONTROL OF ADIPOSE STEM CELLS FOR USE IN TISSUE ENGINEERING**

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Generation of artificial tissues by tissue engineering is highly dependent on the use of cells with high viability and functionality. Cell viability may be influenced by the isolation process used to harvest the cells, as well as by sequential culturing and cell passaging, which could impair cell viability. Although mesenchymal cells are one of the major sources of stem cells in tissue engineering, the cell viability levels of these cells have not been properly determined before generation of artificial tissues. In this work, we analyzed cell viability of cultured rabbit adipose stem cells in order to determine the most viable cell passage to generate artificial bone-like tissues for animal studies of cleft palate regeneration.

Primary cultures of rabbit adipose stem cells were established from small adipose tissue biopsies obtained from the inguinal fat of the rabbit. Adipose tissue was incubated in collagenase to enzymatically digest the extracellular matrix and isolated cells thus obtained were cultured in DMEM medium with fetal bovine serum and antibiotics. Once subconfluent, each cell passage was trypsinized and subcultured during eight consecutive passages. Evaluation of the cell viability was carried out by trypan blue exclusion methods and live/dead® combined assays (calcein AM and ethidium homodimer 1) on cells corresponding to all subcultures analyzed.

Our results showed that cell viability was dependent on the cell subculture analyzed. Analysis of cell viability among the eight passages revealed that the percentage of live cells was always above 86%, with the higher viability corresponding to the 6<sup>th</sup> and 7<sup>th</sup> cell passages and the lower viability at passages 1<sup>st</sup> and 8<sup>th</sup>. These results imply that rabbit adipose stem cells should be preferentially used for the construction of a bioengineered bone substitute for cleft palate treatment using the 6<sup>th</sup> and 7<sup>th</sup> cell passages.

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## **B.17 GENERATION OF ARTIFICIAL BONE TISSUE FOR CLEFT PALATE THERAPY**

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Cleft palate is a congenital malformation with an incidence of between one and two in 1000 live births. Surgical management is complex, since the palate defect must be covered with surrounding tissues, which are not easily available in most cases. In addition, restoration of palatal integrity is usually carried out by using oral mucosa and other soft tissues, without the use of bone or bone substitutes of the palatal bone. In this work, we developed a substitute of the palatal bone using stem cells and biomaterials, and we evaluated this bone substitute at different levels.

Primary adipose stem cells (ADSCs) cell cultures were generated by enzymatic digestion of small adipose tissue biopsies of 8 New Zealand white rabbits. ADSCs were cultured in DMEM medium for expansion. Then, these cells were transdifferentiated to the osteogenic cell lineage by using specific bone-inductive media during 2-3 weeks. Then, a three-dimensional artificial bone was constructed by using fibrin-agarose nanostructured biomaterials with transdifferentiated cells immersed within, and these artificial tissues were cultured for 7, 14, 21, 28 and 35 days *ex vivo*. As controls, artificial bone substitutes with non-transdifferentiated cells were used. Histological analysis was carried out using hematoxylin-eosin staining (H&E), alizarin red and osteocalcin detection in control and transdifferentiated tissues.

Our results demonstrated that both control and bone-transdifferentiated cells efficiently adapted and proliferated into nanostructured fibrin-agarose tridimensional scaffolds from day 7 to day 35. Alizarin red staining and osteocalcin detection showed positive pericellular signal after 28 days of *in vitro* culture only in transdifferentiated cells, but not in control samples.

These results suggest that ADSCs could be efficiently differentiated to bone-like cells which can be used to generate an artificial bone substitute. Further studies should determine if this artificial bone substitute may be useful for surgical reparation of cleft palate in animal models.

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## **B.18 KERATINIZED EPITHELIAL DIFFERENTIATION CAPABILITY OF HUMAN UMBILICAL CORD CELLS AS DETERMINED BY IMMUNOHISTOCHEMISTRY ANALYSIS**

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Human Wharton's jelly stem cells (HWJSC) have inherent differentiation capabilities as compared with other adult mesenchymal cells, showing high expression of typical markers of undifferentiated stem cells such as SOX2, Oct4, CD90 and CD105. However, the epithelial differentiation capability of umbilical cord stem cells has been only attributed to epithelial amnioblasts. In this study, we analyzed several epithelial markers in umbilical cord tissues and in isolated HWJSC in order to understand the epithelial differentiation capability of umbilical cord-derived mesenchymal cells.

HWJSC primary cell cultures were generated by enzymatic digestion of human umbilical cord fragments with trypsin and collagenase I. These tissue cultures were maintained in Amniomax culture medium. Immunohistochemical analyses were carried out for some markers of non-keratinized epithelia (CK7, CK8, CK13) and markers of mature, keratinized epithelia (involucrin) in both formalin-fixed, paraffin-embedded umbilical cord sections and in HWJSC cultures. Cultured human oral fibroblasts were used as controls.

Our results showed that the protein expression of all these markers was very similar in umbilical cord tissues and cultured HWJSC. In general, the expression of markers of keratinized epithelia was more strongly positive than markers of non-keratinized epithelia such as CK13, but all these markers were detected in both tissue types, whereas controls were completely negative.

These results suggest that human umbilical cord-derived mesenchymal cells show some native expression of cytokeratin and involucrin both in situ -in the umbilical cord- and ex vivo -in culture-. In consequence, HWJSC could have intrinsic epithelial differentiation potential, especially to keratinized epithelial types like skin keratinocytes. This supports the use of these cells for the generation of bioengineered epithelial tissues by cell transdifferentiation.

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### **B.19 GENERATION OF BIOMIMETIC SCAFFOLDS BASED ON DETERGENT-DECELLULARIZED CORNEAS USING A BIOREACTOR**

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Construction of an artificial cornea remains challenging mainly due to the difficulties to obtain a functional corneal stroma in the laboratory. The native corneal stroma is composed by keratocytes immersed into highly arranged lamellae consisting of collagen, proteoglycans and several other extracellular matrix proteins. Xenogeneic cornea decellularization could be a proper method to obtain corneal stromas with potential usefulness as corneal xenografts. Several decellularization protocols have been optimized for different tissue types; however, there is no consensus about the best decellularization protocol for the animal cornea. In this work, we have evaluated several detergent-based decellularization protocols on porcine corneas for use in tissue engineering.

Porcine corneas were obtained from a local slaughterhouse. After washing the corneas with PBS and antibiotics, 4 types of detergents were applied independently to the corneas to decellularize them using a specific bioreactor. Sodium-dodecyl-sulfate (SDS), Igepal, Triton-X100 or benzalkonium chloride (BAK) were used at different concentrations (0.01%, 0.05% and 0.10%) and times (12, 24 and 48 hours). After decellularization, porcine corneas were washed with PBS and histologically analyzed.

Histological assays of decellularized corneas showed that all decellularization methods were able to partially remove the cells from the corneas. The endothelial corneal cells were efficiently eliminated with all the protocols applied. Corneal epithelial cells were properly removed with Igepal (48 hours – all concentrations), SDS (0.05% and 0.1% - all times) and triton-X100 (24h and 48h – all concentrations). Stromal keratocytes were completely removed only when SDS 0.05% (24h and 48h) and 0.1% (all times) were used. The integrity of the collagen bundles was partially preserved with all the detergents used.

#### **DISCUSSION**

Our results show that the use of SDS represents the most accurate decellularizing detergent agent for porcine cornea decellularization. This method could have potential usefulness for the generation of corneal scaffolds for clinical or experimental use.

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## **B.20 IN VIVO QUALITY CONTROL OF ARTIFICIAL CORNEAS GENERATED BY TISSUE ENGINEERING**

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Nanostructured fibrin-agarose biomaterials allow the efficient ex vivo generation of bioengineered anterior lamellar corneas. Previous results demonstrated that these corneas could have analogy with the human native cornea, but further in vivo studies should determine the clinical usefulness of these artificial tissues. In this work, we generated a bioengineered anterior lamellar cornea and evaluated this tissue once implanted in vivo.

First, stromal keratocytes and corneal epithelial cells were isolated and cultured from the cornea-scleral limbus of New-Zealand white rabbits. Then, bioengineered anterior lamellar corneas were developed in the laboratory by using a scaffold of human fibrin and 0.1% agarose with keratocytes immersed within and epithelial cells subcultured on top. These artificial corneas were implanted in vivo by anterior lamellar keratoplasty carried out on New Zealand rabbits. The results were analyzed clinically, by optical coherence tomography (OCT) and histologically 6 months after the implant.

Bioengineered anterior lamellar corneas were properly integrated in the host cornea, and no relevant side effects were found in the recipient animals. After an initial adaptation period, optimum optical behavior was found at 6 months follow-up. OCT analysis revealed a regular cornea stroma and epithelium, with a slight interphase at the site of the implant. Furthermore, histological sections showed a well-developed corneal stromal structure where stromal fibers were well integrated. Human keratocytes and epithelial cells showed clear signs of cornea differentiation.

Bioengineered anterior lamellar corneas demonstrated potential usefulness in vivo, with no side effects. The results obtained in this work suggest that these artificial tissues could be clinically useful for the treatment of different pathologies of the superficial cornea.

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**B.21 SEQUENTIAL BASEMENT MEMBRANE DEVELOPMENT IN HUMAN CORNEA, SKIN AND ORAL MUCOSA GENERATED BY TISSUE ENGINEERING**

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The generation of functional epithelial-stromal constructs is strongly dependent on the development of a mature basement membrane. Laminin modulates epithelial cell adhesion, differentiation and migration, and it is the most abundant structural and biologically active component of the epithelium-mesenchyme adhesion system. The aim of the present work was to generate human cornea, skin and oral mucosa substitutes, and to analyze the immunohistochemical expression of laminin.

Primary cell cultures of cornea, skin and oral mucosa epithelial cells and fibroblasts were obtained from human tissue biopsies. Then, fibrin-agarose stromal substitutes were generated with cultured fibroblasts immersed within, and the epithelial cells seeded on top. Hematoxylin and eosin staining, and immunohistochemical analysis of laminin were performed at 1, 2 and 3 weeks of culture.

Histological evaluation of the cornea, skin and oral mucosa constructs revealed the progressive development of the epithelium, and the presence of an integrated and well-formed artificial stroma. Interestingly, an orthotypical pattern of laminin expression first appeared in the artificial cornea after 1 week, and it was evident in the three tissue constructs after 3 weeks of air-liquid interphase culture.

The results suggest that the fibrin-agarose artificial stroma is suitable for the development and maturation of a basement membrane in different complex tissues, such as cornea, skin and oral mucosa.

This work was supported by grants P10-CTS-6060 (proyectos de excelencia) and SAS PI-0273-2010 from Junta de Andalucía, Spain and EC10-285: CAH/Ulc/2010 from the Spanish Ministry of Health, Social Services and Equality.

## **B.22 IN VITRO BEHAVIOR OF HUMAN ORAL MUCOSA FIBROBLASTS CULTURED ON CHITOSAN MEMBRANES AND DESCCELLULARIZED COLLAGEN SCAFFOLDS**

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Several scaffolds have been proposed for tissue repair applications with promising results. These scaffolds should be biocompatible allowing cell proliferation, migration and differentiation *in vitro*. In addition, these biomaterials should be highly biodegradable and mechanically stable *in vivo*. Chitosan and collagen are natural and biodegradable biomaterials, and both have been used clinically to repair periodontal defects. However, the effectiveness of these biomaterials is still controversial. The objective of this work is to evaluate fibroblasts behavior cultured in both chitosan and collagen membranes.

In this work, 10,000 human oral fibroblasts from passage 3 were cultured on the top of both chitosan and collagen membranes. The microscopical analysis using Live & Death methods (calcein AM and ethidium homodimer 1) was carried out at 1, 2, 3 and 4 weeks.

When we analyzed the cell growth and adhesion, we observed a progressive cell growth and adhesion of the fibroblast cultured in the collagen membrane with a low rate of cell death. In turn, fibroblasts cultured on the top of chitosan membrane showed a progressive decrease of cell growth and adhesion, accompanied by high cell death. Our results suggest that the collagen membranes are more biocompatible *in vitro* in comparison with the use of chitosan membrane. The collagen membranes favor progressively the cell viability with a high cellular adhesion, and these results suggest that the collagen membrane could be a better candidate for its use in periodontal repair.

This work was supported by grants FIS PI11/2680 and FIS PI11/2668 from Instituto de Salud Carlos III, Ministry of Economy and Competitiveness, Spain.



### **B.23 DEVELOPMENT OF AN ARTIFICIAL NERVE MODEL USING ADIPOSE STEM CELLS AND FIBRIN-AGAROSE HYDROGELS. AN IN VIVO REGENERATION STUDY**

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The use of neural conduits filled with biomaterials and/or stem cells may improve peripheral nerve regeneration in comparison with the use of hollow conduits. Several types of conduits based on different biomaterials have been reported to promote peripheral nerve regeneration, but a fully efficient neural conduit has not been described to the date. In this regard, the use of highly biocompatible fibrin-agarose scaffolds showed promising results for the generation of artificial human tissues such as the skin, cornea and oral mucosa. The aim of this work is to determine the potential usefulness of fibrin-agarose scaffolds to generate a bioengineered model of peripheral nerve.

A model of sciatic nerve defect was developed by generating a 10-mm nerve gap in Wistar rats. Then, the nerve gap of each animal was repaired using a different neural conduit in each experimental group: 1) NeuraGen® collagen conduit (P-CTR group); 2) collagen conduit filled with acellular fibrin-agarose scaffolds (A-FAH group) and 3) collagen conduit filled with fibrin-agarose containing adipose derived mesenchymal stem cells (ADMSCs) (C-FAH group). After 12 weeks, the animals were euthanized and the implanted conduits were fixed in formalin and paraffin-embedded for histological analysis. The intensity and area-fraction for the positive reaction of the collagen and myelin and for the expression of S100, laminin (LMN) and neurofilament (NFL) were determined.

The differences in the intensity of collagen fibers were significant between the groups C-FAH v/s P-CTR, C-FAH v/s A-FAH and A-FAH v/s P-CTR. No statistical differences were found in the intensity of myelin reaction, but the area-fraction was significantly higher in the group C-FAH v/s P-CTR. In the analysis of the expression of S100, NFL and LMN, we observed significant differences between the groups C-FAH v/s P-CTR and in some cases A-FAH v/s P-CTR.

These results demonstrate the potential clinical usefulness of the cellular fibrin-agarose hydrogels for the treatment of 10-mm gap peripheral nerve defects.

This work was supported by grant IPT-2011-0742-900000 from the Spanish Ministry of Science and Innovation (co-financed by FEDER).

## **B.24 EVALUATION OF THE HISTOLOGICAL PATTERNS OF ARTIFICIAL ORAL MUCOSA SCAFFOLDS WITH INCREASING AGAROSE CONCENTRATIONS**

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Advances in tissue engineering allowed the development of substitutes of human oral mucosa for in vivo and in vitro applications, and several models of artificial human oral mucosa have been developed based in different biomaterials. Characterization and optimization of biomaterials used for the generation of a tissue engineered oral mucosa is a priority and a requirement for clinical use. In this work, we have developed a bioengineered model of artificial oral mucosa stroma using different concentrations of fibrin-agarose biomaterials and we analyzed the histological properties of these bioengineered tissues.

Human stroma substitutes of the human artificial oral mucosa were generated using fibrin-agarose scaffolds with stromal cells immersed within. The concentrations of agarose used were 0%, 0.1% and 0.2%, and these artificial tissues were evaluated after 0, 7, 14, 21 and 28 days of in vitro development. Histological analyses were performed for all samples by Haematoxylin-Eosin staining (H&E), picrosirius, proliferating cell nuclear antigen (PCNA) determination and cortactin detection in order to analyze the cell behavior into the scaffolds. Five gray-scale digital images (2048x1760pixels) were acquired for each type of human oral mucosa construct and each time in culture using a SEM microscopy at 10,000x magnification. All images were analyzed using ImageJ software. Spaces between fibers (empty spaces or 'lakes') were identified and labeled using gray scale thresholding at 70 level (all pixels with a gray level between 0 and 70 were classified as 'lakes'), and the surface area of these 'lakes' within each image was calculated (% 'lakes').

The histological analysis demonstrated that PCNA staining was highly positive for stromal cells immersed in 0% and 0.1% fibrin-agarose scaffolds, especially after 7 days of culture, being only slightly positive for 0.2% scaffolds. Picrosirius staining revealed a homogenous signal in samples corresponding to lower concentrations of agarose. However, 0.2% scaffolds revealed a signal restricted to the pericellular area. The analysis of the cortactin cell migration protein demonstrated that most of the cells immersed in the fibrin-agarose scaffolds were able to migrate into the mesh. Finally, the structural analysis showed that the percentage of lakes tended to decrease after 21 days in the 0.1% and 0.2% fibrin-agarose scaffolds.

These findings suggest that 0.1% fibrin-agarose scaffolds could be adequate biomaterials for human oral mucosa regeneration, supporting adequate levels of cell proliferation, migration and differentiation.

This work was supported by grant FIS PI11/2668 from Instituto de Salud Carlos III, Ministry of Economy and Competitiveness, Spain.

## **B.25 EVALUATION OF THE OPTICAL PROPERTIES IN A MODEL OF HUMAN ANTERIOR LAMELLAR ARTIFICIAL CORNEA**

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Advances in tissue engineering allowed the development of substitutes of human oral mucosa for in vivo and in vitro applications, and several models of artificial human oral mucosa have been developed based in different biomaterials. Characterization and optimization of biomaterials used for the generation of a tissue engineered oral mucosa is a priority and a requirement for clinical use. In this work, we have developed a bioengineered model of artificial oral mucosa stroma using different concentrations of fibrin-agarose biomaterials and we analyzed the histological properties of these bioengineered tissues.

Human anterior lamellar bioengineered corneas were generated in the laboratory using fibrin-agarose biomaterials with keratocytes immersed within and corneal epithelial cells subcultured on top. Once the epithelium matured and stratified, these tissues were optically analyzed by determining the total diffuse reflection and transmission using a single-integrating sphere at increasing developing times. 457.9nm, 488nm, and 514.5nm laser lines were used with an argon laser and 632.8nm using a He-Ne laser. To determine the absorption and scattering coefficients of these corneal constructs, the inverse adding doubling computational method based on the diffusion approximation and radiative transport theory was applied to the diffuse reflection and transmission values.

Histological analysis revealed that the number of cells in the artificial corneas increased with time in culture. Both the absorption and scattering coefficients tended to decrease with increasing wavelength. Time in culture played an important role in the optical behavior of the fibrin-agarose corneal constructs. The absorption properties decreased with increasing time of development in culture, whereas optical scattering increased with time in culture.

These results show that the optical properties of the bioengineered corneas are highly dependent on the cell number, the level of maturation of the epithelial cells and the time of development in culture. This suggests that specific developing times should be selected for applications in which a definite optical behavior is required.

This work was supported by grant FIS PI11/1582 and PI11/2680 from Instituto de Salud Carlos III, Ministry of Economy and Competitiveness, Spain.

## **B.26 X-RAY HISTOCHEMICAL EVALUATION OF CULTURED HUMAN DENTAL PULP MSC**

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Generation of primary cultures of human MSC is always dependent on the cell viability and physiological status of the cultured cells, and only viable cells are suitable for clinical use. However, it is well known that cells cultured during long times are prone to cell death. For that reason, it is necessary to ensure that the cells expanded in GMP facilities are fully viable and a process of necrosis or apoptosis has not been activated before using these cells Tissue Engineering. The aim of this study is to evaluate cell viability and physiological status of human dental pulp stem cells (hDPSC) corresponding to long-term cell cultures to determine if these cells could still be potentially useful for clinical purposes.

Human dental pulp mesenchymal stem cells (hDPSC) were isolated from human teeth using collagenase digestion. Then, cell cultures were maintained during 20 consecutive subcultures using trypsin-EDTA for cell passaging, and passages P11 to P20 were analyzed. To analyze cell proliferation capability, PCNA western-blot was used at each cell passage. To determine cell viability, cells were analyzed by trypan blue exclusion analysis, electron-probe X-ray microanalysis (EPXMA), TUNEL assay and Caspase-4 (pro-apoptotic protein) and BCL7C (anti-apoptotic protein) western-blotting at each cell passage.

Protein determination by western blot showed a progressive PCNA decrease from passage P12 to the end of the study. Interestingly, the percentage of live cells was above 86% at all cell passages analyzed (P11-P20). TUNEL assay detected an increase of apoptotic cells with DNA fragmentation on passages P17 ( $7.21 \pm 2.07$  %) and P19 ( $9.80 \pm 6.80$  %). Caspase-4 levels were low up to passage P14 and increased thereafter. BCL7C did not show significant differences during the study. The K/Na ratio as determined by EPXMA revealed a decrease of cell viability since passage P13 to passage P20. Potassium (K) and chlorine (Cl) intracellular concentrations decreased from P13.

These results suggest that hDPSC kept in culture for long periods (up to 20 cell passages) could still show high cell viability rates, although a progressive reduction if the cell proliferation capability was detected upon cell subculturing. In addition, the TUNEL and western-blotting results show that a process of cell death by apoptosis could be associated to long term subculturing, especially from passage P14, which was confirmed by a sequential K/Na decrease associated to a decrease in the intracellular concentration of Cl. According to these results, hDPSC should be preferentially used until passage P13 with proper cell viability levels.

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## **B.27 MICROANALYTICAL EVALUATION OF NEOFORMED BONE AFTER THE IMPLANT OF BIO-OSS BONE GRAFTS IN THE MAXILLARY SINUS**

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Elevation of the maxillary sinus by using Bio-Oss materials to increase bone thickness is becoming a common technique in dental clinics to prepare the recipient bone for dental implanting. In this work, we will establish a quantitative microanalytical method to evaluate the neoformed bone in order to systematize the microanalytical pattern of the major elements with a role in biomineralization.

Neoformed bone tissue biopsies were obtained from the maxillary sinus of three patients subjected to maxillary sinus elevation with Bio-Oss six months before the study. Each biopsy was cryofixed in liquid nitrogen and cryopreserved at -80°C. Then, specimens were washed in 3% H<sub>2</sub>O<sub>2</sub> for 24h to eliminate the organic component of the bone, air-dried, sputter-coated with carbon in an argon atmosphere and examined in a XL30 Philips scanning electron microscope with an EDAX microanalytical detector. Spectra were collected by pin-point electron beam at 40000X magnification. Twenty analyses were taken per specimen. The peak-to background ratio (P/B) method was used to measure the concentrations of calcium and phosphorus in each sample. Two methods were used for background elimination for the quantitative analysis of the samples: the automatic quantification method and the manual method of background subtraction. Microcrystalline salt standards were used to quantify Ca and P as previously described. All results were calculated as weight fraction percentage of Ca and P.

120 microanalytical spectra were obtained from the neoformed maxillary bone samples analyzed in this work. Our results showed a variable behavior of both Ca and P -the main components of hydroxyapatite- according to the method used, with higher weight fraction percentage of P when the automatic method was used. However, very few differences were found for the quantitative analysis of Ca concentration. The Ca/P index was 3.08 as determined by using the manual method and 2.20 when the automatic method was used. This later index is more similar to the molar index previously described for the hydroxyapatite mineral.

These results reveal that two major elements are predominant in bone tissue at the microanalytical level: P and Ca, the main components of hydroxyapatite. Both elements show a variable ratio, with P or Ca being dominant in each case, which may be explained by the fact that a single point was used at the microanalytical detector. When we compared the values obtained for the weight fraction of P and Ca in neoformed bone, we observed that Ca was predominant in all analyses, independently of the method used for quantification. When the Ca/P index was calculated to determine the relationship between these two elements, we found that this value was close to the values previously described by Riviere and Wheeler (2005) in dental tissues for hydroxyapatite standards.

The most adequate background elimination method for quantitative microanalysis of bone tissues in our material is the automatic method.

Riviere HL, Wheeler HT: Cementum on smilodon sabers. *Anat Rec*285A:634-642, 2005.

This work was supported by CTS-115 (Tissue Engineering Group).

## **B.28 AUTOMATED BIOREACTOR FOR CORNEA DECELLULARIZATION WITH ULTRASONIC SENSORS**

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Automation and control of tissue processes in bioreactors is an open problem in tissue engineering, aimed at creating artificial tissues and organs. To standardize and optimize the process, it is necessary to control most of the parameters that may vary its effectiveness, at the same time that a large number of realizations are carried out in repeatable conditions. To solve this problem, an automated bioreactor with ultrasonic sensors has been conceived, designed and tested. We propose to monitor the changes that may suffer the matrix during the process using mechanical quality parameters.

A bioreactor is designed and produced to effectively solve the need to design and standardize a protocol for decellularization and recellularization for clinical application in a single process. The following parameters need to be controlled in an automated way for the process design in order to ensure repetitive indicators essential to establish specific protocols: (1) time factors, (2) biological factors and (3) mechanical factors. The bioreactor consists of a series of chambers that fulfill incubation conditions: (1) temperature, (2) nutrition time-sequence and quantity, (3) fluid flow velocity and timing, (4) gases, (5) sealing and (6) sterility. The bioreactor includes connected fluid systems for automated control of decellularized agents and culture media for the recellularization process. It incorporates sensors for real-time monitoring of mechanical consistency (ultrasound). The reconstruction of mechanical parameters from nondestructive testing based on ultrasonic transmission and model-based solution of the identification inverse problem is proposed as a novel technique with high potential not only due to the reduced cost, its non-ionizing nature and absence of electric interference. A numerical method to determine the mechanical parameters associated to the process has been developed by combining an inverse problem, applying genetic algorithms to minimize a cost functional, and using a semi-analytical modeling of the interaction between ultrasonic waves and cornea.

Porcine corneas are successfully decellularized by applying a range of different configurations. The quality of the process is validated by histology tests, yielding comparable quality to previous techniques. The evolution of the relevant reconstructed mechanical parameters during the decellularization is validated against histology controls over a timeline. The presented bioreactor can be easily extended to other types of processes and tissues.

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**Keywords:** Decellularization, cornea, monitoring, bioreactor, ultrasonic, tissue engineering.

## B.29 *IN VIVO* EVALUATION OF FUNCTIONALIZED POLYMERIC DRESSINGS FOR ISCHEMIC WOUND HEALING IN AN ANIMAL MODEL

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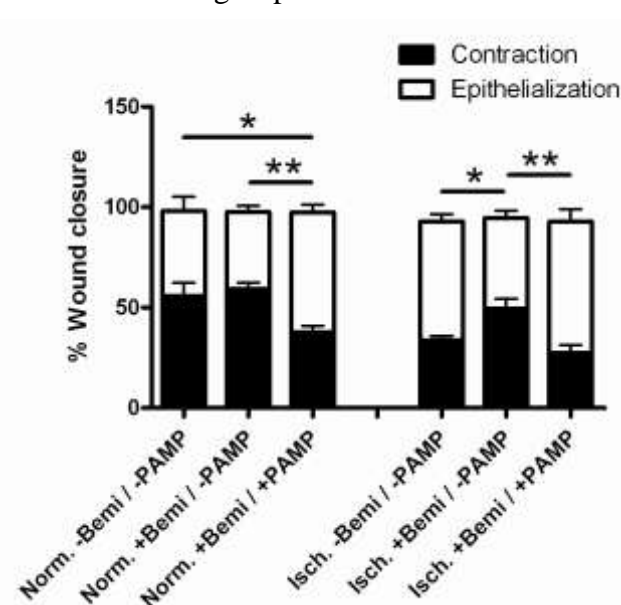
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Vascular ulcers represent an important health issue, being one of the major causes of chronic wounds. Tissue ischemia, a condition associated with this pathology, hampers the normal process of wound healing, leading to a delayed closure or healing failure. The use of polymeric dressings, functionalized with wound healing modulators and angiogenesis enhancers, could improve the outcome of these ulcers. The aim of this study is to evaluate a novel polymeric bilayer dressing functionalized with PAMP (proadrenomedullin N-terminal 20 peptide) and nanoparticles loaded with Bemiparin in an experimental rabbit model of ischemic wound healing.

Composite discs (diameter = 2cm) were prepared, consisting in a segmented-polyurethane membrane (SPU) and a gelatin-hyaluronate hydrogel (HG). Three study groups were set: non-loaded discs (-Bemi/-PAMP), discs with Bemiparin-loaded nanoparticles incorporated in the SPU membrane (+Bemi/-PAMP) and discs with HG functionalized with PAMP and SPU with nanoparticles (+Bemi/+PAMP). Experimental ischemia was performed by ligation of the central artery and vein in the ears of white New Zealand rabbits. Animals (n=18) were divided into two groups: ischemic and normoxic. A full-thickness circular defect (diameter = 2cm) was made in the dorsal side of the ears, and was covered with the three types of dressings. 14 days after intervention, animals were sacrificed and samples were processed for morphometrical, histological and immunohistochemical analyses, in order to evaluate the processes of epithelialization, contraction, angiogenesis and inflammation.

Wound closure percentages were similar in all groups, but a difference in the contribution of the epithelialization and contraction processes was observed. Discs loaded with both PAMP and Bemiparin caused less contraction of the wound and enhanced epithelialization, especially in the ischemic group (Fig. 1). Immunodetection of  $\alpha$ -smooth muscle actin showed an increment in the presence of blood vessels in samples obtained from ischemic animals treated with PAMP and Bemiparin. Both in normoxic and ischemic animals, the higher degradation of the biomaterials and the lesser presence of macrophages was achieved by the +PAMP/+Bemi group.



**Fig. 1.-** Contraction and epithelialization percentages in each study group. (\* $p < 0,05$ ; \*\* $p < 0,01$ )

Polymeric systems functionalized with PAMP and Bemiparin showed an improvement in ischemic wound healing, enhancing reepithelialization and angiogenesis, and modulating the inflammatory process.

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### B.30 HISTOLOGICAL AND MICROSCOPIC EVALUATION OF DENTIN SURFACE AS A BIOLOGICAL SCAFFOLD

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Several histological and microscope techniques are used in the field of tissue engineering as a first step for morphological and image analysis of tissue regeneration studies *in vitro*. On the other hand, dentin is a mineralized structure whose components could have a positive effect on dental pulp stem cells (DPSC) adhesion process, enabling its establishment as a biological scaffold.

The aim of this work was to develop a procedure based on the combination of different staining and microscopy techniques that could allow the analysis of the surfaces and structure of dentin used as a natural scaffold. Additionally, DPSC adhesion and growing was also evaluated.

Dentin cross sections slides were obtained from third human molars indicated for extraction at the Oral Surgery Department from the Dental School of the University of Valencia after the previous informed consent of the patients. Samples were divided into three groups: group 1, dentin treated with 17% ethylenediaminetetraacetic acid (EDTA); group 2, dentin treated with 5.25% sodium hypochlorite (NaClO) and group 3, untreated dentin. Some samples from each group were fixed, decalcified and processed for their analysis by optical microscopy and another group of samples were processed for scanning electron microscopy (SEM).

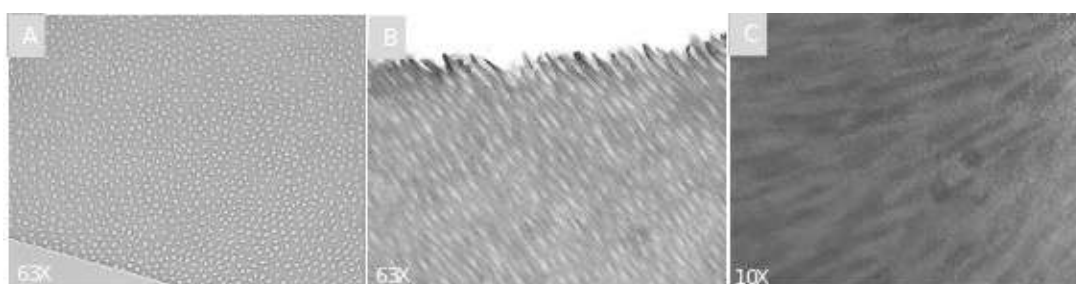
Samples of dentin from the 3 experimental groups were placed in 24-well plates, seeded with DPSC and cell cultured for 3 and 7 days. After assessing cell adhesion and proliferation on dentin surfaces on the whole-mount samples they were fixed, decalcified and processed for optical microscopic analysis.

Optical microscopy of hematoxylin-eosin stained allowed us to observe structural differences between treated and untreated dentin. Group 1, EDTA-treated dentin surfaces showed dentinal tubules (Figure, panels A and B) of a wider diameter than those from groups 2 and 3. Additionally, group 1 dentinal tubules did not conserve an integral structure due to erosion. SEM analysis confirmed these differences.

Microscopic analysis of dentin samples co-cultured with cells and hematoxylin-eosin stained showed the interaction between DPSC and dentin surfaces (Figure, panel C) thus allowing the monitoring of the adhesion of cells at short times of incubation.

Differences were found in EDTA -treated dentin samples compared with samples from groups 2 and 3 as a greater dentinal tubules size and a cleaner surface free of a smear layer, which characteristics could improve the behaviour of dentin as a biological scaffold.

The microscopic analysis of dentin and DPSC interaction can be developed using habitual histological procedures herein described, yielding important information about the adhesion process. The same sample used in the whole-mount analysis can be subsequently processed to obtain paraffin sections that could be analyzed using the habitual histological approaches.





### **B.31 BONE REGENERATION RESPONSE IN RATS' JAWS AFTER THE IMPLANTATION OF PTHRP AND A DIFERENT-WAYS TREATED MESSOPOROUS BIOACTIVE GLASS**

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Nowadays bone loss is considered an important problem by Restorative Dentistry due to the required bone regeneration in jaws in order to secure a correct balance in stomatognathic system. Silica-based bioactive glasses have supplied successful solutions for repairing small to medium bone defects for some time. The high biocompatibility and the specific biological effects of their reaction products (both leached or formed at the surface) after implantation have made bioactive glasses one of the preferential grafts for restoring small bone defects in periodontal, middle ear, maxillofacial and spine surgery ed. as bio-glasses. This response may be facilitated if messoporous bioactive glasses (MBGs) are coated with different co-adjuvanted substances.

The aim of this study is to analyze in healthy and osteoporotic rats models the bone tissue regeneration responses in right jaws surgical defects in which a different ways treated MBG is implanted. These regeneration responses are compared at 4 and 12 weeks after the implantation with it is produced in absence of biomaterial.

A two different-ways treated MBG: White (Si-HAP, silicon-doped hydroxyapatite) and APTS857 [Si-HAP functionalized with (3-Aminopropyl)triethoxysilane]. These biomaterials may be coated or not with PTHrp peptide by immersion in a PBS solution.

60 Wistar rats divided in two groups, healthy (n=30) and osteoporotic (n=30). Each group is divided in six different sub-groups (n=6) corresponding to: Si-HAP, APTS857, Si-HAP+PTHrp, APTS857+PTHrp, sham and sham+PTHrp. Each sub-group have two times of study (n=3), 4 and 12 weeks.

In order to analyze the regeneration response histological techniques as hematoxylin-eosin staining, TGF $\beta$  and RunX2 immunochemistry are used. For the statistical study GraphPad Prism 5.01 software is used.

In absence of scaffold, both with healthy and with osteoporotic rats, defects are not repaired at 12 weeks due to the big size of the defect. The addition of PTHrp in the defect (with no bio-glass) facilitates fast bone regeneration and the restoration of the bone table.

Si-HAP, in healthy rats and at 4 weeks, is observed in the superficial outer area and encapsulated in a dense fibrotic layer that continues with granulation tissue coming from periodontal ligament. At 12 weeks the bio-glass in different degradation levels is observed above in the external surface above new- one formation areas. The addition of PTHrp accelerates the induction of new bone formation. New bone formation even at 4 weeks and much higher at 12 weeks. In osteoporotic rats this bio-glass, whether PTHrP is added or not, does not produce an appropriate bone regeneration, noticing low quality new-bone tissue with decalcification areas and osteoblastic hypertrophy. Nonetheless this regeneration process is better in cases when periodontal ligament in involved.

APTS857 in healthy and osteoporotic rats produces an important osteogenic response that is recognizable even at 4 weeks and noticing the bio-glass encapsulated in the outer area above new-bone bordering bio-glass rests. The addition of PTHrp accelerates this process in healthy rats but not in osteoporotic.

The different-ways treated MBG produce good bone regeneration responses that are faster and better in the cases PTHrp is added except with APTS857 in osteoporotic rats. This improvement is also noticed when periodontal ligament is involved.

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### **B.32 DETERMINATION OF BONE DENSITY AND ISQ VALUE OF THE ESSENTIAL CONE 1'5 KLOCKNER IMPLANT. CONVENTIONAL MODEL VS NEW PROTOTYPE**

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The macroscopic design of a dental implant can stimulate different cellular specific answers according to its characteristics. It also has effect on dental implant primary stability and the ability to hold its charge during or after osseointegration. The correct design choice improves and keeps periimplant marginal bone avoiding the generation of high stress concentration at the implant neck area and consequent crestal bone resorption. Study objective is to determine the causal relationship between the change of narrow essential cone 1'5 Klockner implant core diameter and loops design and the biomechanical optimization of the bone to implant interface, by the determination of the bone density (BDd) and the ISQ values measurements.

8 Essential Cone 1'5 Klockner internal connection dental implants were placed in each right hemimaxilla and hind tibiae of 4 Adult Minipig females of 90kg weight and 10 months of age, switching different platforms (3'5 And 4 Mm) Of The Standard Model Of This Implant With A New Prototype (Core 0'2mm wider and more sharp turns). The animals were sacrificed 4, 15, 28 And 42 days after the implant insertion, measuring the osstell isq value at the time of sacrifice. histologic study was performed by scanning electron microscopy (SEM). BD was determined using the image analysis program image j. the program graphpad prism 5 was used to perform the statistical analysis, comparing bd results in hind tibiae (cortical bone) to maxillary (cancellous bone) in short (4 and 15 days) and long (28 and 42 days) term after implant insertion, in addition to the BD percentages for the standard model and the new implant prototype.

A significant difference ( $p < 0.0001$ ) was demonstrated between short term BD in hind tibiae respect to the of the hemimaxilla, also proven by comparing the ISQ values, while there is no difference between the BD associated to the different implant models in this phase. However, at long term, BD associated to the conventional implant model is greater than the one of the new implant prototype.

1) The 0'2mm increase of the implant core in addition to the more sharp turns design does not help to keep a greater bd at the bone-to-implant interface of the new implant prototype. 2) The conventional essential cone implant model of klockner presents a greater bd percentage than the new model. 3) There is a correlation between ISQ measurements and BD.

### **B.33 OSTEOCONDUCTIVE POTENTIAL OF BARRIER PLGA MEMBRANES FUNCTIONALIZED WITH NANOMETRIC SILICA THIN FILMS DESIGNED FOR BONE TISSUE REGENERATION**

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New biomaterials have been developed to stimulate the bone tissue formation by promoting the osteoblast proliferation and differentiation, to overcome the growing needs of bone replacement therapies. The challenge of surface functionalization in biomaterials intended to be used as barrier membranes leads to render them bioactive, able to trigger a specific cell response. We have developed a model in which PLGA membranes, intended to be used as barrier membranes for bone guided regeneration, have been functionalized. We describe a new approach consisting of the deposition at room temperature by plasma enhanced chemical vapour deposition (PECVD) of a very thin layer of SiO<sub>2</sub> on the surface of PLGA membranes (SiO<sub>2</sub>/PLGA).

HOB<sup>®</sup> human osteoblasts seeded on a series of PLGA and SiO<sub>2</sub> functionalized PLGA membranes, were daily examined and immunolabelled after 24, 48 and 72 hours for actin cytoskeleton and focal adhesion development, with rhodamine phalloidin and anti-vinculin FITC conjugate. As size and shape of cell spreading area, as well as the number, size, shape and distribution of focal adhesion plaques are decisive for further migratory, proliferative and differentiation behaviour of anchorage-dependent cells, an image-based quantitative feature extraction design was performed. The PLGA membranes were prepared from a 1.5 wt% PLGA dichloromethane solution by evaporation of the solvent on a teflon plate, SiO<sub>2</sub> was deposited onto the PLGA membranes by PECVD in a plasma reactor with a remote configuration, and a 15 nm layer of SiO<sub>2</sub> was deposited on the polymeric substrate, where static contact angles of water were determined by the Young method. Test surfaces were exposed to UV light for 20 min each side, under sterile conditions prior to cell seeding.

Osteoblasts grown on the SiO<sub>2</sub> functionalized PLGA membranes showed defined stress fibers formation, with a clear polarization of actin cytoskeleton towards strongly evident vinculin positive sites, which defined a clear osteoblast orientation at any experimental time. Focal adhesions appeared to be significantly more numerous and well developed in those cells grown on the SiO<sub>2</sub> functionalized membranes than in bare PLGA membranes. The exploratory factor analysis performed on morphometric variables considered (area, perimeter, circularity, aspect ratio, roundness and solidity) rendered three morphometric indexes (“solid roundness”, “stretch” and “size”) that significantly related to cell changes associated with SiO<sub>2</sub> functionalized PLGA membranes.

Both the morphological changes and the focal adhesion development identified in our human osteoblast model significantly related to cell growth on SiO<sub>2</sub> treated PLGA, functionalized with the novel Si deposition method developed by our group. Focal adhesion quantification and actin cytoskeleton arrangement were selected as useful markers of the osteoconductive potential of the substrata. The methodology described provides a biocompatible and durable functionalization on PLGA devices that elicits a significant osteoblast response and, in addition, stands the standard sterilization protocols. These findings present a clinical translational potential, resulting by now in the development of a patented “resorbable membrane for Guided Bone Regeneration” (patent number P201232018)

#### Acknowledgments

We thank the Junta de Andalucía (Projects P09-CTS-5189, and Instituto de Salud Carlos III (FIS PI 09/00508) for financial support

### **B.34 ADHESION AND DIFFERENTIATION OF DENTAL PULP STEM CELS ON DENTAL SURFACES**

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Human dental pulp stem cells (hDPSC) are able to differentiate into dental tissues. Therefore, hDPSC have been postulated as a valuable option in tissue regeneration therapies. Mineralized dental components and their tridimensional structure may have an effect on hDPSC differentiation.

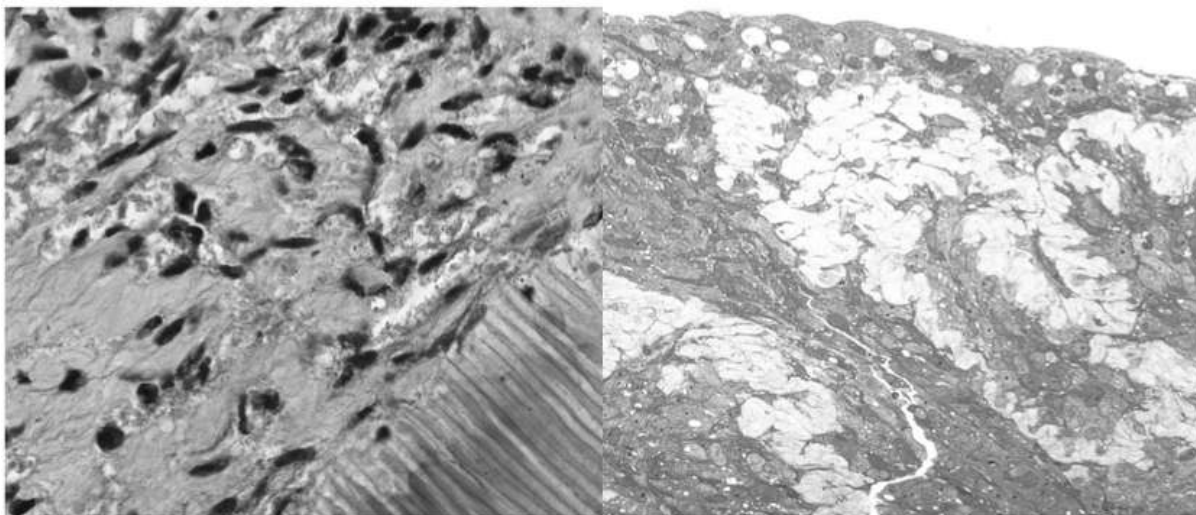
The aim of our work was to compare the adhesion, proliferation and differentiation capacity hDPSC on three different surfaces: dentin with tubules perpendicular to the surface, dentin with tubules parallels to the surface and cementum.

The hDPSC were obtained from young patient's third molars and phenotyped by flow cytometry. They were cultured on dental root cross sections at different times (from 1 to 6 weeks) in basal medium with and without ascorbic acid to induce collagen synthesis. Cell adhesion, proliferation, secretory activity and possible differentiation were analyzed using optical microscopy and transmission electron microscopy (TEM).

At days three and seven it was observed a great proliferation and adhesion of cells around dentin and cement using a phase contrast microscope, although after paraffin embedding this adhesion resulted to be unstable.

At week six, cells were observed forming dense and complex layers completely surrounding the dental fragments with a strong and stable adhesion at this time.

The hDPSC adopted different morphologies on each of the three surfaces: on dentin with tubules perpendicular to the surface hDPSC adopted a pseudo-odontoblast morphology, emitting prolongations within tubules with a low production of matrix; on hDPSC dentin with tubules parallels to the surface and cementum formed flat layers with a high production of matrix between them and the corresponding surface.



### B.35 TISSUE ENGINEERING: CHITOSAN FOR ARTICULAR CARTILAGE REGENERATION

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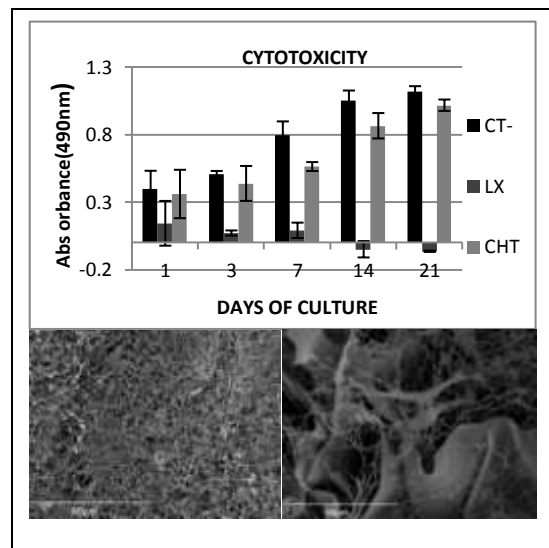
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Cartilage pathologies have a high incidence in the world's population. Numerous studies point to tissue engineering as better alternative for the repair and regeneration of articular cartilage by using biomaterials. Chitosan has been proposed as an adequate biomaterial for regeneration of cartilage. The aim of this study is to assess the behavior of Chitosan coated with platelet-rich plasma (PRP) as a biodegradable scaffold material for cartilage regeneration.

Primary cultures were performed with human chondrocytes obtained from healthy regions of the articular cartilage of donor patients undergoing resection. Isolated chondrocytes were cultured and their chondral nature was determined immunohistochemically.

Cytotoxicity study was carried out by culturing such cells with extracts of chitosan over 3 weeks, using latex exposure (positive control) and basic culture medium (negative control) with MTS cell proliferation assay. Once discarded the toxic effect of this material on chondrocytes, the cells were settle on macroporous chitosan discs (3 millimeters in diameter and 1 millimeter in height, with 90 micron diameter pores), that were previously coated with platelet-rich plasma, using CaCl<sub>2</sub> as a coagulant. Cells performance were studied in parallel by optical and electron microscopy techniques.

The cytotoxic effect of chitosan along the culture period was discarded. Similar behavior was observed between cells exposed to chitosan and those exposed to control medium, however cells exposed to latex showed an absence of cell proliferation and marked cytotoxicity. Immunohistochemical technique determined the presence of aggrecan and type II collagen, which indicated that the cells belong to the chondrocytic lineage. After chondrocytes were seeded over the chitosan scaffold covered with fibrin, cell adhesion and proliferation were studied with optical and electron microscope. That revealed the presence of a thin fibrillar mesh covering the chitosan scaffold, over which chondrocytes were observed. Chitosan is not toxic to the chondrocytes. Platelet-rich plasma is a positive candidate for adhesion of chondrocytes in the chitosan scaffold.



### **B.36 MORPHOLOGICAL STUDY OF CHANGES PRODUCED BY HISTOPATHOLOGICAL GRAPHENE OXIDE GRAFTED IN RATS.**

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Graphene oxide (GO) is a material known to have numerous biomedical applications in the near future.

The purpose of the study was to evaluate morphologically the tissue reaction induced by GO grafted in an experimental model.

We performed 5 mm calotarian defects and dorsal intramuscular "bags" in a total of 20 male Wistar rats that were grafted with GO, and maintained for 21 days (n = 10), and 5 months (n = 10). We performed a morphological and morphometric studies evaluating the cellular response (fibroblasts, monocytes/macrophages, giant cells, inflammatory infiltrate, vessels, granuloma formation) per mm<sup>2</sup>.

The diameter of calotarian defects was reduced to  $2.25 \pm 0.34$  mm in GO group, and  $2.29 \pm 0.48$  mm (p = 0.841) in the unfilled defects at 21 days and  $1.91 \pm 0.4$  mm vs.  $1.86 \pm 0.5$  mm at 5 months (p = 0.819), respectively. In no animal was observed complete closure of the defect or the presence of new bone between the sheets of GO. The tissue reaction was composed of granulation tissue with fibroblasts, neo-formed capillaries and a varying ratio inflammatory cell, highlighting monocytes/macrophages and multinucleated giant cells forming granulomas after 5 months with persistence of GO particles.

The tissue response versus GO is the formation of granulation tissue and granulomatous response without interposition of newly formed bone, that does not interfere with the bone repair process.

### **B.37 HISTOLOGICAL ANALYSIS OF IN VIVO OSTEOGENESIS FROM MESENCHYMAL STEM CELL IMPLANT GENETICALLY MODIFIED WITH BMP2/7.**

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One of the main challenges in the area of implant for bone regeneration is obtaining a material that accelerates ossification after severe bone lesions, which is capable of achieving adequate bone density to preserve the original form and architecture. For this purpose, various technologies have been tested that involve stem cells, natural and synthetic scaffold materials, various bone growth factors, and others. **PURPOSE.** Evaluate by histological analysis and immunostaining, the osteogenesis of an implant consisting of stem cells genetically modified for expression of BMP 2 and 7, embedded in a demineralized bone matrix (DBM) in a sheep model.

A three component (I-3C) implant comprised of mesenchymal stem cells (MSCs), transduced *ex vivo* with an adenoviral vector that expresses a combination of the bone morphogenetic proteins 2 and 7 (AdBMP2/7), embedded in a DBM was generated. This Implant was tested in a sheep (*Ovis aries*) model in a weight-bearing lesion. The implant was placed in the area of a lesion created by complete osteotomy with a distraction of the mid-shaft of the tibia. Three experimental groups were tested: Control 1 without the implant (WI), control 2 with the MSC implant (MSC-I); and the 3-component implant (I-3C). After 10 weeks of follow-up, the sheep were sacrificed to extract the treated bone areas. These were fixed with formalin-glutaraldehyde, and were decalcified with 10% formic acid for two weeks followed by 2N HCl for 24 hours. The samples were processed for paraffin embedding; sections obtained were stained with H-E, Masson trichrome, and immunostaining for collagen I and II. In the WI group, the periosteum had thickened and the bone cortex was composed mainly of trabecular bone with areas of dense fibrous tissue. Inside the samples, fibrosis was abundant with little spongy bone; there was no hyaline cartilage and amorphous and fibrillar material dominated. Also, infiltration by lymphocytes, plasma cells, and some macrophages was observed. The MSC-I group presented thickening of the periosteum, and cortical bone tissue showed formation of highly vascularized fibrocollagenous tissue. There were small areas of hypertrophic hyaline cartilage in the center of the lesion and large areas of fibrous tissue. In the I-C3 Group, the periosteum was thin and slightly fibrous with abundant differentiating stellate and fusiform cells. Abundant spongy bone with good tissue consolidation with a uniform and compact matrix was present, with no cartilage. There was a greater proportion of compact bone tissue. The immunohistochemical reactions type I collagen were positive in the three groups and for collagen II, it was very low in the focal areas of hyaline cartilage in the MSC-I group.

These findings demonstrate that the quality of the newly formed bone was greater in the three component implant group with better consolidation of bone tissue, in contrast with the non-implant group and the MSCs without genetic modification.



**Keywords:**

Histological osteogenesis, Mesenchymal stem cell, Implants modified.

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**B.38 TIME-DEPENDENT BIOACTIVATION OF PECVD Ti NANOLAYERED PET, BY ULTRAVIOLET LIGHT ILLUMINATION, ENHANCES SCAFFOLD OSTEOCONDUCTIVITY. AN IN VITRO HUMAN OSTEOBLASTS MODEL.**Salido M<sup>1</sup>, Terriza A<sup>2</sup>, Vilches JI<sup>1</sup>, de la Orden E<sup>1</sup>, González-Caballero JL, Vilches J<sup>1</sup>

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Osteoblasts are sensitive to a number of input signals from the surrounding microenvironment acting as mechanical, chemical and topographical cues, and leading to a wide number of signal transduction and responses. We herein present an in vitro model designed to prove the effect of UV illumination in activating the surface of amorphous TiO<sub>2</sub> supported layers to enhance their osteoconductivity. The role on HOB® cells grown on functionalized PET samples of the changes in the wettability of PECVD Ti activated surfaces, that could result in a superhydrophilic transformation have been assessed *in vitro*.

We have assessed HOB cells response to u.v. sterilized PET scaffolds, clean or activated with PECVD Ti nanolayers, and the possible influence of time-dependent bioactivation of the samples after u.v. irradiation on HOB cells growth. TiO<sub>2</sub> was deposited on the PET substrate plates by PECVD in a plasma reactor. The samples were characterized by XPS to determine their surface chemical state and composition, AFM for surface topography and by water CA measurements to check the wetting characteristics of surfaces. Samples were u.v. light sterilized prior to cell seeding. Half the number of the samples was immediately seeded with HOB® cells, while the remaining half was stored under dark ambient conditions for up to 4 weeks, prior to cell seeding. HOB® human osteoblasts grown on test surfaces were immunolabelled with rhodamine phalloidine and monoclonal anti-vinculin FITC conjugate, after 24,48 h and 7 days in culture. Cells were image analysed to assess surface influence on cytoskeletal organization, focal adhesion number and development and cell morphology.

HOB cells seeded immediately after irradiation of TiO<sub>2</sub> activated substrata substantially improved cell attachment, growth, polarization and differentiation and focal adhesion contact development with respect to those seeded on the samples that were kept in dark. These latter showed similar results to clean PET surfaces at some experimental times. Another interesting finding is that a relatively inactive PET turned to be bioactive by PECVD surface deposition of a very thin layer of photoactivated TiO<sub>2</sub>

We have developed a reproducible cell culture protocol including a successful sterilization of TiO<sub>2</sub> activated PET samples and the preservation of both the polymeric scaffold integrity and the TiO<sub>2</sub> layer preservation. The methodology proposed can be a valuable tool that could be applied to different polymeric substrates. The u.v. irradiation protocol proposed induces wettability changes on PECVD Ti activated surfaces that result in a superhydrophilic transformation<sup>1</sup>. The combination of PECVD Ti activated samples with an adequate u.v. exposition effectively stimulated osteoblast spreading and attachment. The major time dependent variations on the cellular response to the surface changes induced by UV photofunctionalization supports the careful observance of time dependent changes on bioactivity as an important factor for improving u.v. sterilized samples suitability for clinical purposes.

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<sup>1</sup>Terriza A, Díaz-Cuenca A, Yubero F, Barranco A, Gonzalez-Elipe AR, Caballero JLG, Vilches J, Salido M. 2013. Light induced hydrophilicity and osteoblast adhesion promotion on amorphous TiO<sub>2</sub>. *J Biomed Mater Res Part A* 2013;101A: 1026–1035.

## C. Human Genetics

### **C.1 MICRONUCLEUS TEST: CYTOME ASSAY, CANCER AND HEALTH BIOMARKER**

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Micronuclei are structures similar to a nucleus but small size and localized in the cytoplasm. Micronucleus contents are chromosome fragments or whole chromosomes. From the eighties, the micronucleus test is considered a biomarker for early induced genetic damage. Micronucleus test with cytochalasin B (CBMN test) is used to evaluate genotoxic effects induced by physical and chemical environmental agents, and to estimate the genetic damage induced in population groups exposed to such agents. After the study of large groups of control population, it is estimated that CBMN test can be used as a marker of health status with ability to establish a cancer risk.

Finally, recent studies about the onset of cancer, based on single catastrophic events able to originate a cancer, such as chromothripsis or chromoanagenesis, are going to understand through an anomalous replication of DNA within the micronucleus.

## C.2 THE ONCOPROTEIN SRSF1 REGULATES LIG1 MRNA EXPRESSION IN LUNG CANCER

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Lung cancer remains the leading cause of cancer deaths worldwide with an overall 5 years survival rate of 10-15%. It is divided in two main histological types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is the most common lung cancer histology (80-85%) and includes three major histologic subtypes: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. The low survival rate is mainly due to the fact that two thirds of lung cancer patients are diagnosed in an advanced stage at which curative treatment is not yet available. Early identification of the disease could potentially favor the use of therapeutic interventions associated with prolonged survival. Therefore, further biological characterization of lung carcinogenesis is required to improve prognosis, therapy or prediction of response to specific drugs.

In recent years, the relevance of RNA metabolism has increasingly been recognized in a variety of diseases, including cancer. Some studies have shown that, in lung cancer, the concentration, composition, localization or activity of RNA binding proteins (RBPs) is altered. Modifications in the levels of RBPs elicit changes in the profile of mRNA expression of cancer-related genes. Likewise, SRSF1 is an mRNA modifier protein which regulates several steps during mRNA processing. Moreover, it is an oncoprotein with roles in the establishment and maintenance of cell transformation.

In the present work, we examined the role of SRSF1 in the regulation of relevant functions of NSCLC cells. SRSF1 expression was inhibited by small interfering RNAs and a microarray platform was used to detect cancer related genes which expression is regulated by SRSF1. Our results showed that DNA ligase 1 (LIG1) is regulated by SRSF1 in a post-transcriptional manner. We studied the expression of SRSF1 and LIG1 at mRNA and protein levels in lung cancer cell lines and primary tumors. A strong correlation between both proteins was observed. Furthermore, siRNA mediated LIG1 inhibition reduced the proliferation rate and the ability to form colonies in cellular models corresponding to the three histological subtypes of NSCLC.

Finally, we evaluated LIG1 protein expression in primary tumors to determine its correlation with clinicopathological variables and assess its prognostic significance. We found that high LIG1 expression was associated with shorter progression free survival in adenocarcinoma patients ( $p=0.009$ ).

In conclusion, these results show that SRSF1 and LIG1 have a relevant role in lung carcinogenesis. Specifically, LIG1 promotes lung cancer cell proliferation and its expression predicts poor prognosis in lung adenocarcinoma.

### C.3 CANCER PROTECTION ELICITED BY A SINGLE NUCLEOTIDE POLYMORPHISM CLOSE TO THE ADRENOMEDULLIN GENE

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The lifetime risk of developing cancer is regulated by genetic variants, including polymorphisms. Characterizing such variants may help in developing protocols for personalized medicine. Adrenomedullin (AM) and proadrenomedullin N-terminal 20 peptide (PAMP) are regulatory peptides coded by the proadrenomedullin gene, *adm*. These peptides induce tumor cell growth, migration, angiogenesis, immune surveillance evasion, and reduce apoptosis, thus being involved in cancer promotion and progression. Carriers of a single nucleotide polymorphism (SNP) in the proximity of the *adm* gene have lower levels of circulating peptides. The aim of the present work is to investigate whether carriers of this SNP (**rs4910118**) are protected against cancer. To this end, we performed a retrospective study with specimens obtained from the Carlos III DNA National Bank (University of Salamanca, Spain). DNA samples included 238 breast cancer patients, 348 lung cancer patients, 474 cardiac insufficiency patients, and 500 healthy donors of advanced age ( $70 \pm 10$  years). All samples were genotyped using mismatch amplification mutation assay (MAMA) and confirmation was achieved by direct sequencing. The main outcome measure was the minor allele frequency (MAF) for each group. Pearson's Chi-square was used to compare SNP frequencies. Only fourteen samples (out of 1,560) had the minor allele (Table 1), with a MAF in healthy donors of 0.90 %. The number of cancer patients carrying the minor allele was statistically significantly lower than that of healthy donors (odds ratio = 0.216, 95% C.I. = 0.048-0.967,  $p = 0.028$ ). In conclusion, carriers of the minor allele have a 4.6 fold lower lifetime risk of developing cancer than carriers of the major allele. Interestingly, the MAF for this SNP is much larger among the Chinese population (23.4 %) suggesting that this polymorphism, among others, may be responsible for the differential rates of cancer prevalence found among races and geographical regions. Knowing the **rs4910118** genotype may be useful for stratifying patients in clinical trials and to design prevention strategies.

Table 1. Results of the genotyping of patients and donors.

	Genotype*			Total
	C-C	C-T	T-T	
<b>Healthy controls</b>	492	7	1	500
<b>Heart failure</b>	470	4	0	474
<b>Cancer (total)</b>	584	1	1	586
<b>Breast cancer</b>	237	0	1	238
<b>Lung cancer</b>	347	1	0	348

\* Genotypes are expressed as C-C for major allele homozygotes, T-T for minor allele homozygotes, and C-T for the heterozygotes.

#### **C.4 MELATONIN BIOSYNTHESIS IN THYROID C CELLS IS REGULATED BY TSH**

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In previous studies our group demonstrated that C cells functionally express thyrotropin receptors and could, therefore, be involved in the hypothalamic-pituitary-thyroid axis. More recently, our group reported that C cells synthesize melatonin and suggested a paracrine role for this neurohormone and natural antioxidant molecule in the regulation of thyroid activity. Based upon these results, in the present study we have analyzed the effect of TSH in the regulation of the enzymatic machinery for Melatonin biosynthesis in C cells.

The expression of key enzymes for melatonin biosynthesis (AANAT, HIOMT) was studied by real-time qRT-PCR in a rat C-cell line (CA77) after treatment with 10nM TSH for 24h. The same studies were carried out in thyroid tissue from rats treated with TRH for 5 weeks.

AANAT and HIOMT mRNA levels are significantly decreased after treatment with TSH and TRH. Cytoplasmic immunoreactivity for AANAT was observed in CA77 and rat thyroid C-cells, thus confirming RT-PCR results.

The finding that melatonin is synthesized by C cells under TSH control supports the hypothesis that this endocrine population not only plays a role in calcium homeostasis but it may also be involved in controlling follicular-cell micro-environment and suggests a paracrine role for melatonin on thyrocyte activity.

## D. Reproductive Biology

### D.1 COMPOSITION AND MATURATION OF THE ZONA PELLUCIDA

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Fertilization is a process that involves the participation of two highly specialized cell types: sperm and oocyte. The discovery of the intimate mechanism that regulates this process is of special relevance for human and animals. Here, some biological aspects of a specialized extracellular matrix that surrounds the mammalian oocyte, called the zona pellucida (ZP), are analysed. The ZP is responsible for specific sperm binding and induction of the acrosome, and participates in blocking polyspermy. Mouse ZP has been described as being composed of only a few glycoproteins, named ZP1, ZP2 and ZP3.

These glycoproteins are detected as broad bands when they are separated by SDS- PAGE electrophoresis due to their glycosylation heterogeneity, which makes it difficult to separate the different proteins. However, the use of proteomic analysis overcomes this handicap and, by this means, an additional protein (ZP1-4) has been identified in the human and rat ZP. We recently reported four proteins in the hamster and rabbit ZP.

Here, we present strong evidence supporting the existence of four ZP proteins in the cat and some marsupials. All these data suggest that species containing four ZP are more common than was previously expected. Future experiments are necessary to understand the consequences of an additional protein in the ZP structure and function. It is generally accepted that glycosylation of the ZP proteins is responsible for sperm binding. We have observed a different glycosylation pattern in the ZP of different species that could be involved in the species specificity of the gamete interaction. Thus, some of the carbohydrates detected are unique to human ZP. Additionally, this glycosylation contributes to the complexity of the ZP. For example, a heterogeneous distribution of the carbohydrate residues throughout the thickness of the ZP was detected. Finally, a zona maturation responsible for the ZP changes produced during folliculogenesis was described. To investigate this process a lectin-gold histochemistry was performed showing a differential carbohydrate composition in the ZP of different ovarian follicles. However, the ZP is also modified after ovulation during oocyte transit through the oviduct. This oviductal maturation of the ZP has not been analyzed in detail due to the difficulty involved in isolating the oviductal oocytes. Recently, it was observed that ovarian oocytes incubated with oviductal fluid showed increased monospermy during the *in vitro* fertilization process. This greater efficiency was attributed to the binding of the oviductal glycoprotein OVGPI. Other oviductal proteins such as SPAM1 could affect gamete maturation and the fertilization. In summary, current knowledge on the ZP composition and changes during ovarian and oviductal maturation is presented.

This study is supported by the Spanish MICINN (AGL2012-40180-C03-02), European Commission (FEDER / ERDF) and Fundación Séneca (0452/GERM/06).



## D.2 PARTIAL LOSS OF GLYCOCONJUGATE CONTENT DURING ACROSOME BIOGENESIS IN A STERILE GOPC DEFICIENT MOUSE

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Fertility is a serious problem of reproductive health and in approximately one-half of the cases the reason is male infertility [1]. Some of them are caused by genetic alterations [2], thus mutant sterile mice can be useful models for human male infertility. The acrosome, an organelle which develops over the anterior half of the head in sperm cell and contains hydrolytic enzymes, is essential for the fertilization. The acrosome biogenesis is a highly complex process that involves the transport of protein and membrane from the Golgi apparatus to the developing acrosome.  $GOPC^{-/-}$  mutant mice show abnormal heads (globozoospermia) with fragmented acrosomes [3].

We have analyzed the acrosomal glycoconjugate composition in wild type and  $GOPC^{-/-}$  mutant mice by means of lectin cytochemistry. Testis were fixed and processed routinely for both light and electron microscopy. The lectins employed recognized N-Acetyl-Glucosamine (WGA, DSA, sWGA), sialic acid (WGA, MAA-II and SNA), N-Acetyl-Galactosamine (SBA, DBA, HPA and MPA/MPL), Fucose (UEA-I, AAL and LTA), Mannose (ConA, GNA and LCA), Galactose (PNA, BPL-BPP, MAA-I, BSI-B4 and RCA-I), and complex bisected glycans (PHA-L).

We have found some differences in acrosomal glycoconjugate composition between  $GOPC^{-/-}$  knockout and wild type mice. While MAA-I, LCA and LTA lectins labeled the acrosome of wild type mice, the mutant was negative. Then, some Galactose-, Mannose- and Fucose-containing glycans are missing in the mutant acrosome. In addition, the acrosome labeling of WGA, SBA, HPA and MPA/MPL was weaker in the mutant mice, suggesting a minor expression of sialic acid- and N-Acetyl-Galactosamine-containing glycoconjugates in the acrosome of the  $GOPC^{-/-}$  mouse (fig. 1). Since GOPC protein is involved in vesicular traffic [3], these results suggest a deficiency in some glycoconjugate transport between the Golgi apparatus and the acrosome during the spermiogenesis in  $GOPC^{-/-}$  mice, which causes a partial loss in the glycoconjugate content of the acrosome.

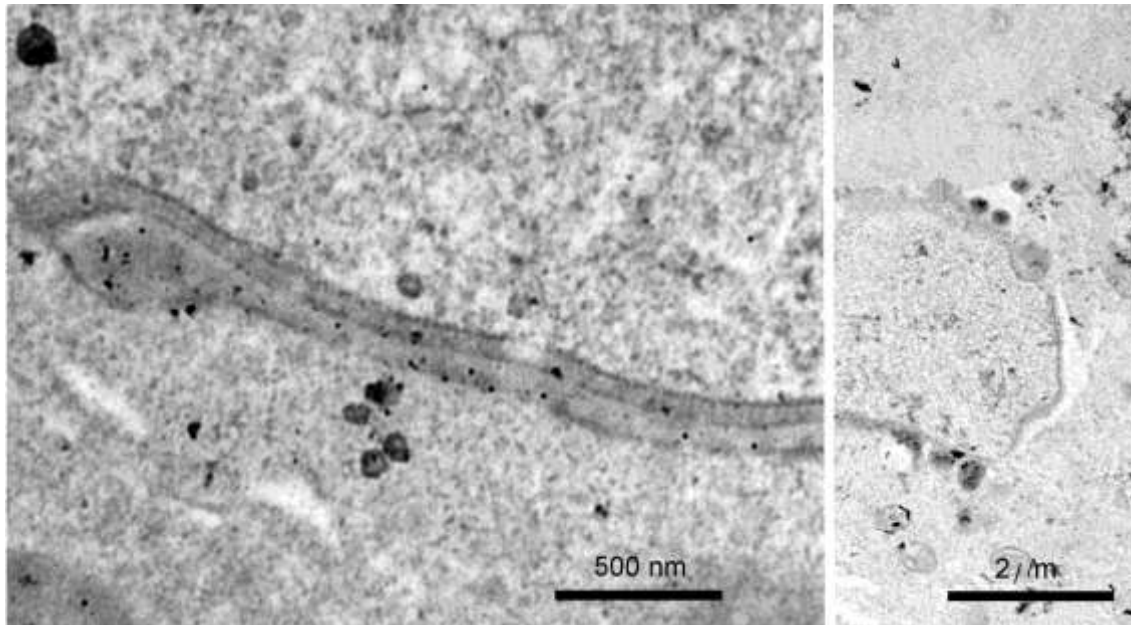


Fig 1. SBA-colloidal gold-labeling of round spermatids in wild type (left) and  $GOPC^{-/-}$  (right) mouse, as observed by electron microscopy. No labeling was shown in the fragmented acrosome of the mutant mouse.

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### **D.3 PROLIFERATION OF SPERMATOGONIA AND SERTOLI CELLS DURING TESTICULAR RECRUDESCENCE OF SYRIAN HAMSTER AFTER EXPOSURE TO SHORT PHOTOPERIOD**

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During the seasonal reproduction of Syrian hamster, there is a period of testicular regression caused by short photoperiod. During this testicular regression period, the reduction in proliferative activity of the spermatogonia, accompanied by germ cell and Sertoli cell apoptosis, causes the reduction of the seminiferous epithelium. After regression, testicular recrudescence occurs spontaneously, bringing with it restoration of the seminiferous epithelium and complete spermatogenesis. The present communication aims to determine whether there are changes in the proliferative activity of spermatogonia and Sertoli cells during testicular recrudescence in the Syrian hamster after exposure to short photoperiod. A total of 24 hamsters were used (21 treated and 3 as controls). The treated animals were subjected to an 8:16 light-dark photoperiod while the control animals were subjected to a 12:12 light-dark photoperiod. Seven treated animals plus one from the control group were sacrificed at 16, 19 and 21 weeks., Testes were fixed in methacarn and embedded in paraffin. The proliferative activity was determined by proliferating cell nuclear antigen (PCNA) and the proliferation index (PI) of spermatogonia and Sertoli cells was calculated. The spermatogonia/Sertoli cell ratio was also determined. Three recrudescence groups were established: Initial (IR), Advanced (AR) and Total (TR). The spermatogonia PI was significantly higher in these three groups compared with the control group, and no significant differences were found between the three groups. The PI of Sertoli cells was significantly higher in IR and AR than in the control. The spermatogonia/Sertoli cell ratio was significantly lower in IR than in TR and the control group. In conclusion: a) A constant increase in the proliferative activity of spermatogonia take place throughout the recrudescence process; b) this proliferative activity is accompanied by a constant increase in the proliferative activity of Sertoli cells maintaining the spermatogonia/Sertoli cell ratio; c) minimal proliferative activity of Sertoli cells is observed in Syrian hamster in normal conditions; d) this proliferative activity increased during testicular recrudescence such as occurs in postnatal development showing, both biological processes, high levels of FSH and d) our results agree with others that say that the Sertoli cell is not always a quiescent cell in mammals.

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#### D.4 HYPOTHALAMIC-HYPOPHYSEAL-GONADAL AXIS ACTIVITY IN THE SOUTH AMERICAN PLAINS VIZCACHA DURING GESTATION

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The correct operation of the hypothalamic-hypophyseal-gonadal (HHG) axis is essential to ensure fertility and reproductive efficiency. The gonadotropin releasing hormone (GnRH) is the key regulator of the HHG axis. GnRH pulsatility activates hypophyseal function resulting in delivery of the gonadotropins, follicular stimulating hormone (FSH) and luteal hormone (LH). Both hormones stimulate ovarian folliculogenesis and luteolysis with increments on estradiol (E<sub>2</sub>) and progesterone (Pg) secretion. Then, both steroid hormones act over the hypothalamus modulating GnRH synthesis and delivery. Estrogen Receptors  $\alpha$  and  $\beta$  (ER  $\alpha$  and ER  $\beta$ ) are able to induce GnRH expression acting as transcription factors directly on GnRH promoter specific sites or non-directly stimulating the transcription of progesterone receptors (PR) which can in turn induce GnRH expression. In most mammals, this cycle repeats from puberty to menopause except in pregnancy. Previous studies showed that the South American plains vizcacha (*Lagostomus maximus*) ovulate up to 800 oocytes per reproductive cycle, and show folliculogenesis progressing to pre-ovulatory stages with a possible ovulatory event at mid-gestation. The aim of this work was to analyze hypothalamic ER  $\alpha$  ER  $\beta$  and PR expression through gestation in the vizcacha, to relate it with GnRH and LH secretion, and with the functional state of the ovary in gestating females. We used plains vizcachas at early- and mid-pregnancy to investigate hypothalamic GnRH and serum LH, E<sub>2</sub> and Pg levels, and GnRH, ER  $\alpha$  ER  $\beta$  and PR hypothalamic localization and expression, by ELISA, RIA, Immunohistochemistry, Immunofluorescence and Western-blot. Hypothalamic immunolocalization of ER  $\alpha$  ER  $\beta$  and PR was observed in neurons of preoptic area (PA), supraoptic nucleus (SON) and median eminence (ME), with similar distribution among groups, whereas, GnRH was localized in somas and axonic varicosities at the same regions. In addition, ER  $\alpha$  ER  $\beta$  and PR were co-localized in the same neurons, while only a few neurons of preoptic area showed co-expression of PR and GnRH. The analysis of ER  $\alpha$  ER  $\beta$  and PR expression throughout pregnancy showed that ER  $\alpha$  and PR significantly varied during gestation ( $p < 0.05$ ) while ER  $\beta$  did not show significant variations. ER  $\alpha$  and PR showed a similar pattern with 32% and 30% increment in mid-pregnant vizcachas with respect to early-pregnant females respectively. The study of hormones during gestation showed significant increments ( $p < 0.05$ ) in the levels of GnRH (60%), LH (700%), Pg (328%) and E<sub>2</sub> (356%) at mid-gestating when compared with early-pregnant animals. These results suggest that mid-gestating vizcachas show a neuroendocrinological hormonal pattern concordant with ovulation but with elevated levels of Pg did not seem to suppress GnRH expression. In addition, ER  $\alpha$  and PR seem not to be inhibited by high levels of their specific hormones during gestation. Altogether, these observations may indicate a special modulation of its HHG axis.

## **D.5 CUMULUS OOCYTE COMPLEXES (COCS) COLLECTION FROM ADULT HOUSECAT (*FELIS CATUS*) OVARIES IN REPRODUCTIVE SEASON AND SEASONAL ANESTROUS**

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The domestic cat (*Felis catus*) is the only one of the thirty-seven species of the *Felidae* family who is not a threatened or endangered species and one of the few who lives in society. It is characterized by estrous cycle associated with increased daily light hours in temperate countries, presenting several cycles along the same season (seasonal polyestrous). It is currently being used as a successfully experimental model in reproductive technologies for application in wild felines threatened or endangered.

The purpose of this study was to obtain cumulus oocyte complexes (COCs) from the ovaries of adult domestic cat in breeding season and seasonal anestrous and compare the results.

The ovaries were obtained from young females over a year old in good nutritional status without hormone treatment. The animals were from the urban area of the city of Montevideo, and were ovariectomized in the owner's home by professional veterinarian. Twenty-six ovaries were obtained from thirteen adult domestic cats in spring breeding season months (October-November) and thirty-two ovaries from sixteen adult domestic cats in seasonal anoestrus late autumn months (May-June). Both groups of ovaries were transported to the laboratory within three hours after its extraction in 0.9% saline solution and refrigerated at 4°C. The ovaries were fragmented by microdissection in Petri dish with phosphate-buffered saline solution (PBS) modified with inactivated fetal calf serum (FCS) and antibiotics (penicillin-streptomycin). Subsequently the ovaries were evaluated under a stereoscopic microscope and good quality COCs were selected (dark homogeneous cytoplasm, full integrity of pellucide zone and several layers (3-5) of cumulus cells) and feasible to be used in reproductive technologies protocols such as *in vitro* maturation, cryopreservation, etc.

We retrieved four hundred forty-two (442) good quality COCs from breeding season ovaries cats with an average of seventeen (17) COCs per ovary and three hundred thirty four (334) COCs with similar characteristics from seasonally anestrous ovaries cats, corresponding to an average of ten (10) COCs per ovary. There were not morphological differences between COCs obtained from cats in breeding season with those obtained from seasonally anestrous cats.

Therefore the analysis of the results allows us to conclude that the number of good quality COCs obtained per ovary was greater in those from cats in breeding season compared to the number of COCs from ovaries obtained by seasonally anestrous cats. Continuing studies in our laboratory seek to complete this finding.

## **D.6 MORPHOLOGICAL AND MORPHOMETRIC CHANGES IN TESTES OF HAMSTER MESOCRICETUS AURATUS DURING TESTICULAR RECRUDESCENCE AFTER EXPOSURE TO SHORT PHOTOPERIOD**

Sánchez-Huertas MM<sup>1</sup>, Seco-Rovira V<sup>1</sup>, Beltrán-Frutos E<sup>1</sup>, Madrid JF<sup>1</sup>, Sáez FJ<sup>2</sup>, Ferrer C<sup>1</sup>, Canteras M<sup>3</sup>, Quesada-Cubo V<sup>1</sup>, Pastor LM<sup>1</sup>

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Syrian hamster's seasonal reproduction is mediated by photoperiod. When it is shortened, the testes undergo a regression, in which its size decreases and it suffers important alterations in both the interstitium and seminiferous tubules, leading to sterility. Subsequently, testes undergo a phase of recrudescence that allows the full development of spermatogenesis with a consequent recovery of fertility. In the present communication, we study the recrudescence process, focusing on a histological description as well as on the morphometric changes observed. A total of 24 hamsters were used (21 treated and 3 controls). The treated animals were subjected to an 8:16 light-dark photoperiod while the control animals were subjected to a 12:12 light-dark photoperiod. Seven treated animals plus one from the control group were sacrificed at 16, 19 and 21 weeks. Testes were fixed in methacarn, embedded in paraffin and stained with hematoxylin-eosin. Three recrudescence groups were established: Initial (IR), Advanced (AR) and Total (TR). Image analysis was used to determine the following parameters: tubular diameter; tubular and interstitial volume, seminiferous tubule length and seminiferous epithelium volume. The proportion of tubular sections according to histological type was also determined in each group, identifying the following types: normal, hypospermatogenic, tubular sections with spermatocyte arrest and tubular sections with spermatid arrest. On the one hand, all the variables were significantly higher in the AR and TR groups compared with the IR group while, on the other hand, the values obtained in TR were similar to those found in the control group. As regards the proportion of normal tubular sections, this was similar between the controls and AR and TR. In IR, no normal tubular sections were found. In IR the proportion of hypospermatogenic sections were significantly lower and the proportion of sections with spermatid arrest was significantly higher compared to the other study groups. In conclusion: a) histologically, the recrudescence process represents a restart of spermatogenesis, which is already complete half way through the process; b) the seminiferous tubule gradually grows in length and diameter, allowing the volume of the seminiferous epithelium which it contains to increase in size.

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### **D.7 MORPHOMETRIC CHANGES IN SYRIAN HAMSTER (MESOCRICETUS AURATUS) TESTES DURING TESTICULAR REGRESSION AFTER EXPOSURE TO SHORT PHOTOPERIOD**

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The Syrian hamster is a seasonal breeding animal whose non-breeding season coincides with the period of short days. It has been seen that in completely regressed animals, profound morphological changes take place which lead to total atrophy of the testis. In this communication we study morphometric changes that take place in the seminiferous epithelium of Syrian hamster during testicular regression. A total of 50 Syrian hamsters (45 treated, 5 controls) were used. The treated groups were submitted to an 8:16 light-dark photoperiod for 12 weeks. The following morphometric variables were studied by image analysis: means tubular diameter (MTD), mean epithelial area (MEA), testicular (Vt), tubular (Vst) and interstitial (Vit) volumes, length of seminiferous tubule (Lst) and the volume of the seminiferous epithelium (Vse) establishing 3 regression groups - mild (MR), strong (SR) and total (TR). The results showed a significant decrease in MTD, Vt, Vst, MEA and Vse in all three regression groups compared with the control, until reaching the total regression. However, Vit and Lst only suffered a significant decrease during the later stages of regression and not from the outset. In conclusion: a) at the onset of testicular regression there is a strong decrease in the size of testicular structures, primarily affecting tubular diameter and testicular volume. b) In contrast, Vit and Lst asynchronously decrease in advanced stages of testicular regression. c) According to these results, testicular volume loss would occur in two phases, the first mainly due to tubular contraction as a result of the loss of the seminiferous epithelium, while in a second phase shortening of the seminiferous tubule would be accompanied by a decrease in Vit.

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## D.8 GLYCOCONJUGATE CONTENT IN THE ACROSOME OF TNP2 -/- KNOCKOUT MOUSE

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Recently, several knockout mice with abnormal spermatogenesis have been produced[1]. One of them is defective for the transition nuclear protein-2 (TNP2), involved in the histone replacement by protamines during chromatin condensation which occurs during spermiogenesis. Tnp2<sup>-/-</sup> mice can also have defective acrosomes[2].

We have looked for the presence of glycoconjugates in both wild type and knockout Tnp2<sup>-/-</sup> mice by means of lectin cytochemistry at both light and electron microscope. Twenty one different lectins recognizing different types of glycoconjugates were tested: N-Acetyl-Glucosamine (WGA, DSA, sWGA), sialic acid (WGA, MAA-II and SNA), N-Acetyl-Galactosamine (SBA, DBA, HPA and MPA/MPL), Fucose (UEA-I, AAL and LTA), Mannose (ConA, GNA and LCA), Galactose (PNA, BPL-BPP, MAA-I, BSI-B4 and RCA-I), and complex bisected glycans (PHA-L).

We have only found little differences in lectin labeling between the acrosomes of wild type and knockout mice.

Acrosome of mutant animals was more strongly labeled by SBA, MAA-I, LCA and DSA than wild type ones, suggesting a higher concentration of N-Acetyl-galactosamine-, Galactose-, Mannose- and N-Acetyl-Glucosamine-containing glycans in the knockout. In addition, SBA and MAA-I labeled both round and elongated spermatids in the knockout mice, while in the wild type only labeled to round spermatids.

The acrosome of wild type animals was negative to BSI-B4, a lectin which labeled the acrosome of Tnp2<sup>-/-</sup> mice (fig. 1). This indicates the presence of some Gal-containing glycans in the spermatid of knockout, which are absent in the wild type animal.

Finally, the LTA lectin, which recognizes Fucose, labeled the acrosome of round spermatids but not elongated spermatids in wild type, while the acrosome of elongated spermatids but not round spermatids was labeled in knockout mice.

These data suggest that the deregulation of nuclear condensation and protein expression during spermiogenesis could produce alterations in the normal acrosome biogenesis



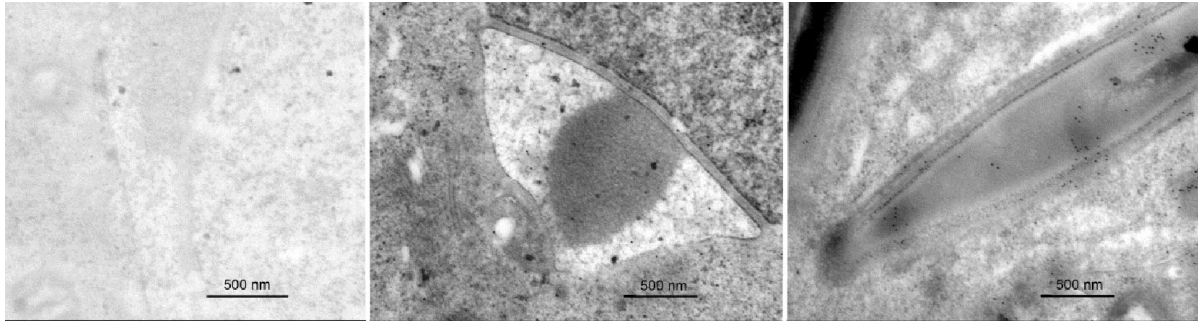


Figure 1. Electron micrographs of round spermatids from wild type (left) and knockout mice (center), and elongated spermatid from a *Tnp2*<sup>-/-</sup> mouse (right) labeled by BSI-B4. The presence of Galactose-containing glycans is showed in the acrosome of both round and elongated spermatids of mutant mouse, but no labeling was seen in the wild type mouse.

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## E. Neurohistology

### **E.1 INVOLVEMENT OF THE NEUROVASCULAR UNIT IN ALZHEIMER'S DISEASE AFTER STROKE. DONOR LA419: POSSIBLE STRATEGY FOR TREATMENT**

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Neurovascular unit dysfunction contributes to ischemia, cognitive disorders and neurodegeneration, including progression of chronic disorders of the CNS such as Alzheimer's disease (AD). When the neurovascular unit function is affected, in addition to a decreased oxygen and metabolite supplies, the amyloid beta protein clearance is compromised which favors the progression of the disease. Thus, the search for effective therapies for the prevention and / or treatment of neurovascular unit dysfunctions is a matter of basic and practical interest.

In this study we have performed permanent focal ischemia by occluding the middle cerebral artery (pMCAO) in a mouse model for AD: double transgenic bearing human mutations associated with early-onset AD (APP<sup>swe</sup>-PS1<sup>dE9</sup>). So, we have tested a nitric oxide donor developed by LACER SA laboratories, the compound LA419, as a potential candidate for the neurovascular unit dysfunction.

The product LA419 is a new generation organic nitrate which acts through the eNOS signaling pathway. Its antioxidant, anti-ischemic, antithrombotic, and antiatherosclerotic properties at doses that did not induce hemodynamic changes that alter blood pressure, have been well described. The drug was administered intraperitoneal in APP/PS1 mice 15 minutes after infarction.

Our results indicate that LA419 decreases the infarct volume in double transgenic mouse 3 months years old submitted to MCAO compared to the untreated one. This decrease not occurred in the same experimental model with 12 months years old.

We conclude that administration of LA419 can be considered a possible clinical strategy for treatment of diseases that course with neurovascular affectionation.

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## **E.2 HYPOTHERMIA PREVENTS ANGIOGENESIS DEVELOPMENT IN A MODEL OF HYPOXIC ISCHEMIC RETINOPHATY**

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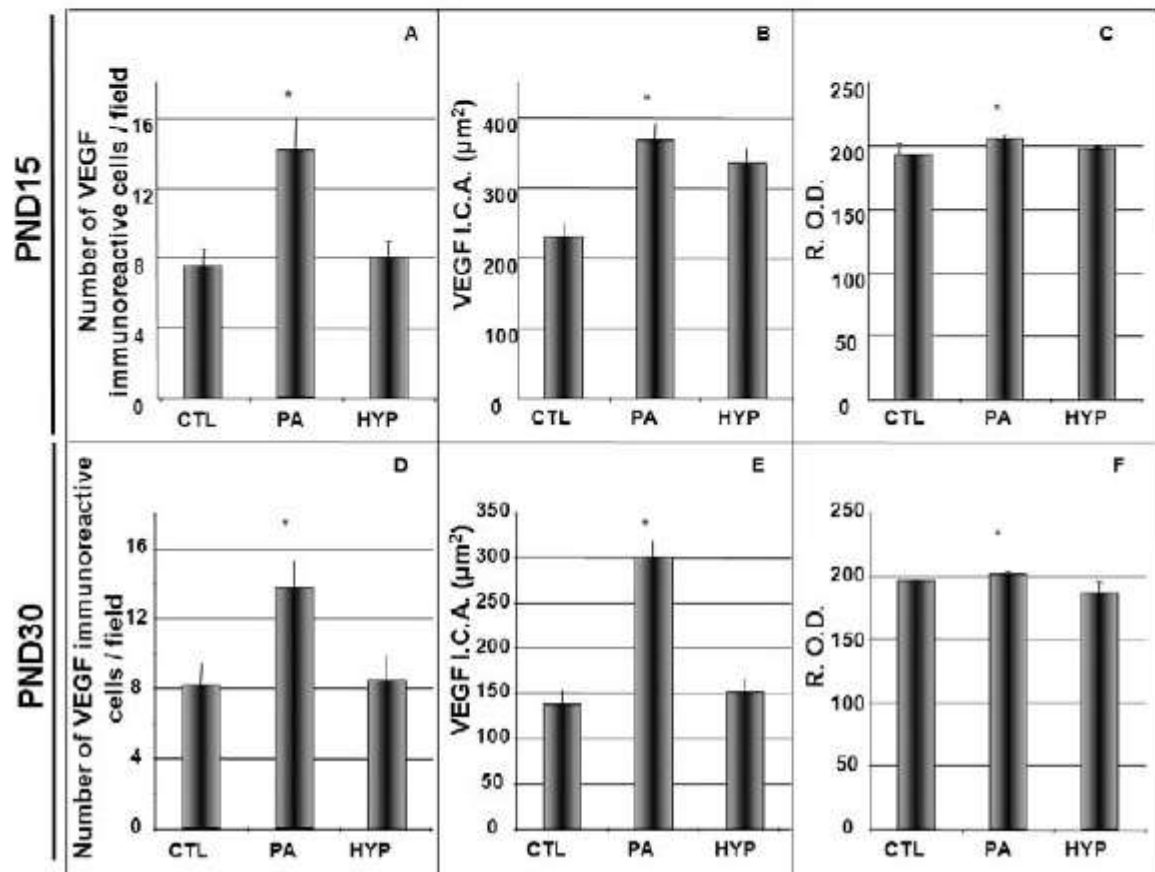
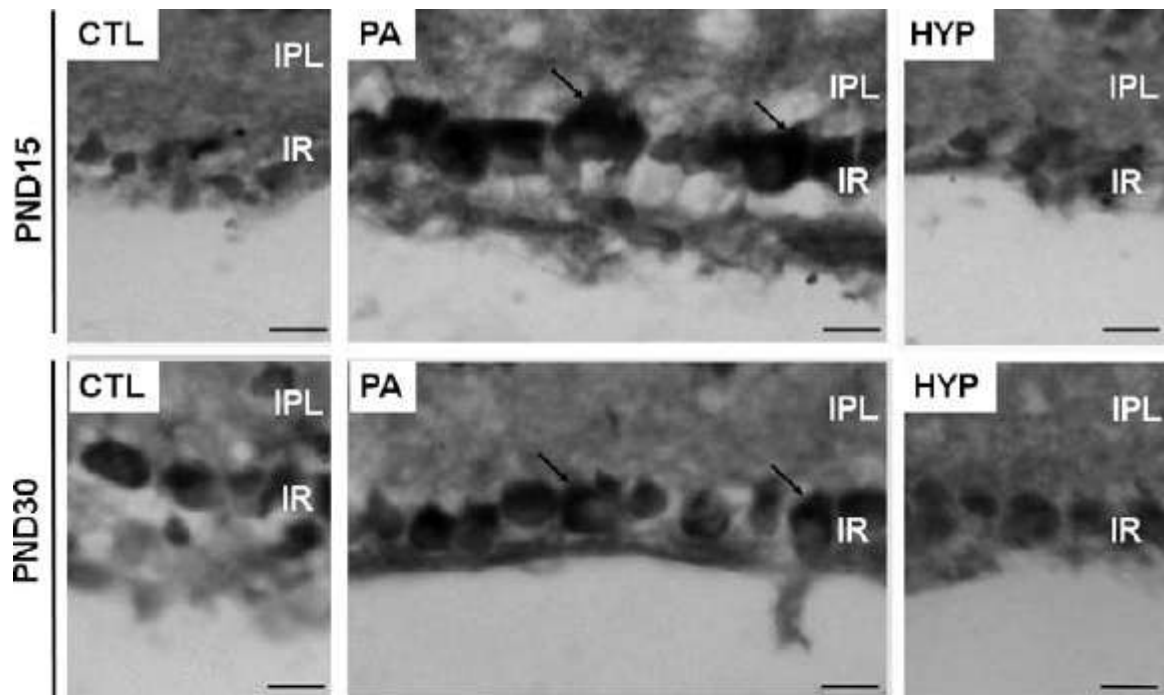
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One-third of asphyctic neonates develop long-term neurological injuries, including several degrees of hypoxic ischemic retinopathy which includes neurodegeneration, gliosis and angiogenesis. Our purpose was to perform a time course study of the neovascularization induced as a response to perinatal asphyxia. In addition, we evaluated hypothermia as a possible protective treatment. We used retinas of 6 hour-old and 7, 15, 21 and 30-day-old male Sprague-Dawley rats that were exposed to perinatal asphyxia at either 37°C (PA) or 15°C (HYP). Born to term animals were used as controls (CTL). We evaluated: 1) the thickness of the most inner layers of the retina (IR), including internal limiting membrane, the retinal nerve fiber layer, and the ganglion cell layer, with routine H&E staining and tomato lectin histochemistry, 2) adrenomedullin (AM), and VEGF by immunohistochemistry, immunofluorescence, and Western blotting, and 3) HIF-1 $\alpha$  and HIF-1 $\beta$  protein expression by Western blotting. We observed a significant increment in IR thickness in the PA group from postnatal day (PND) 15 on. This alteration was concordant with an increased number of new vessels stained with tomato lectin. In PA animals, AM expression was significantly increased from PND7 on. Morphometric studies of AM and VEGF expression showed significant increments in the PA group from PND15 on in IR and ganglion cells respectively. In addition, we observed colocalization of AM and GFAP in the internal limiting membrane, in the perivascular glia of the IR and in the inner processes of Müller cells. At 6 hours, HIF-1 $\alpha$  expression was significantly increased in PA animals as compared to CTL, similarly HIF-1 $\beta$  expression in the PA group was also increased. The retinas of the HYP group did not show differences when compared with CTL at any age. In conclusion, this work suggests that PA induces an early increment in HIF-1 that could enhance AM and VEGF expression generating an aberrant angiogenesis with increased thickness of the IR. In addition, hypothermia treatment showed a strong protective effect.

VEGF immunohistochemistry



### **E.3 NEUROPROTECTIVE EFFECT OF RESVERATROL PRETREATMENT IN NEONATAL RAT BRAIN AFTER HYPOXIA-ISCHEMIA**

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One of the most common causes of mortality and morbidity in children is perinatal hypoxia-ischemia (HI). Resveratrol (3,5,40 -trihydroxystilbene) is a phytoalexin found in grapes that has been reported to decrease oxidative stress and attenuate inflammation. The aim of the present work was to evaluate the effect of resveratrol administered 10 minutes before or immediately after HI brain injury in neonatal rats using the Rice-Vannucci model.

Seven-day-old (P7) Sprague-Dawley rats were randomly assigned to four different experimental groups: Control (n=8), HI (n=8) and HI treated with resveratrol 10 minutes before (RvtB, n=8) and immediately after (RvtA, n=8) the injury. While pups without injury were used as controls, the three hypoxic-ischemic groups were subjected to permanent ligation of the left common carotid artery and then asphyxiated for 2 hours and a quarter with 8% O<sub>2</sub>. Treated animals received a single intraperitoneal injection of 20 mg/kg resveratrol (diluted in DMSO and in saline solution) 10 minutes before or immediately after the HI event. Seven days after surgery (P14), brains were collected and coronal sections were Nissl-stained for histological evaluation of the infarct area. In order to evaluate the damage in different brain areas a semi-quantitative neuropathological scoring system was used.

HI animals presented an infarct area in the ipsilateral side with loss of brain tissue. Moreover, asphyctic animals showed swollen neurons in the parietal cortex and in the CA1 and CA2-CA3 areas and in the dentate gyrus of the hippocampus. Even though resveratrol pretreated animals revealed a mild damage in the ipsilateral side of the brain, no infarcted areas were observed and the damage was significantly lower compared to HI group. Regarding to the quantitative analysis of the infarct area by Image J program, a high percentage of damage is showed in HI group in comparison with Control and RvtB. RvtA revealed also a severe percentage of tissue loss. The semi-quantitative neuropathological scoring system demonstrated a damage located at the level of hippocampus and parietal cortex of ipsilateral hemisphere in HI group. Protection effect by resveratrol injected 10 minutes before the injury was evident when injury was assessed by neuropathological scoring, but resveratrol did not protect when is injected just after.

Our results suggest a neuroprotective effect of resveratrol in neonatal brain after hypoxia-ischemia by reducing infarct volum when administered before injury but not when administered immediately after HI.

Acknowledgments: This work was supported by grant from the Basque Government IT 773/13 and BFI-2011-129.

#### **E.4 ROLE OF MELATONIN ON THE NO/NOS SYSTEM IN A MODEL OF BRAIN ISCHEMIC-HYPOBARIC HYPOXIA**

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Nitric oxide (NO) is a versatile diffusible signaling molecule with physiological actions involved in neuromodulation, immune response, and vasodilatation. In addition, NO appears to be key in brain hypoxic-ischemic injury. Many studies have demonstrated a neuroprotective effect for melatonin based on its antioxidant activity. We analyzed the response of NO system in the brain of rats submitted to a combined model of ischemia and hypobaric hypoxia (I+HH) followed by two reoxygenation periods of 0 and 2 hours (0h and 2h). In addition, we studied the effects of melatonin in such model. Specifically, we determined: 1) the amount of NO<sub>x</sub> (nitrate/nitrite and S-nitroso compounds), 2) the expression of eNOS and nNOS isoforms and 3) the damage caused by protein nitration.

Our results show that the administration of melatonin does not affect NO<sub>x</sub> levels, but implies an increase in eNOS expression after 2 hours of reperfusion. On the other hand, nNOS expression suffers from an earlier increase after ischemia (0h) in melatonin-treated animals. In relation to nitrated protein detection, a decrease was observed after the administration of melatonin in both reoxygenation periods. In summary, the administration of melatonin in our model of brain hypoxic-ischemic seems to modulate the NO system response.

Supported by MICINN (SAF2008-03938).

## **E.5 PROADRENOMEDULLIN N-TERMINAL 20 PEPTIDE (PAMP) INCREASES KINESIN VELOCITY**

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Vesicles and other organelles move inside the cell through a transport system based on microtubules and motor proteins. At this time, little is known about intracellular factors that regulate motor speed.

Here we show that proadrenomedullin N-terminal 20 peptide (PAMP) is an intracellular peptide that regulates kinesin velocity and its ATPase activity in a dose-dependent manner.

The terminal amide group of PAMP is required for this activity and the smallest peptide fragment retaining a kinesin modulatory role is PAMP<sub>(12-20)</sub>. The peptides were active on a variety of kinesins, including kinesin-1, chromokinesin, CENP-E, and Eg5. PAMP increased the speed of microtubules gliding on immobilized kinesins, thus identifying PAMP as an intracellular peptide that regulates kinesin velocity. Furthermore, mouse hippocampal neurons lacking PAMP displayed a mitochondrial transport defect.

Since many of the current cancer chemotherapies target the microtubules and their associated proteins, PAMP may constitute a new molecular target for cell division regulation. In addition, these results may be also relevant for the rapidly evolving discipline of nanotechnology since PAMP may be used as an accelerator of nanodevices. PAMP is the first peptide shown to be involved in the regulation of kinesin velocity. The implications of this observation for basic biology and nanotechnology warrant future studies in this field.

## E.6 INTERSTITIAL CELLS IN CAJAL'S SLIDES NOWADAYS PHOTOGRAHIED AND ASSESSED

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In the last chapter of his book "*Textura del Sistema nervioso del hombre y los vertebrados*" Cajal presents his most interesting observations about the innervation of pancreas and digestive tract where he first describes a particular type of cells: Interstitial Cajal's cells (ICCs).

With the aim of accurately approaching to Cajal's descriptions and interpretations about ICCs, those original slides kept in the Cajal Institute of Madrid have been photographed.

Pictures have been taken of a total of fourteen slides corresponding to pancreas and intestine. The preparations still keep the original labelling, made by Cajal himself; some of them were marked with a "b", when the preparations were good ones, and some others marked with "bb", when they were considered as very good. Some of the labels show technical notes like "diluted with alcohol 1/3", or even little sketches indicating the precise location of some cells. Some areas of the preparations are a bit faded, but the slides are generally in a relative good state of preservation. However, as the sections were thick, most of them appear ridged, and it was necessary to focus on a small area at a time in order to perform reconstructions from several pictures. Afterwards, those pictures taken nowadays and original drawings have been compared.

These cells were defined by Cajal as: "small, fusiform or triangular cells white little protoplasm that had a number of varicose anastomosed processes". He located them around intestinal nervous plexus and pancreas: "They are abundant and spread all over the pancreas lying between acini, their prolongations being accommodated to its convexity".

One of the ideas contributed by Cajal but forgotten by most of the contemporary authors consists of the relationship between ICCs and blood vessels, as it is shown in his wonderful slides. Another important concept was the consideration of these cells as primitive neurons.

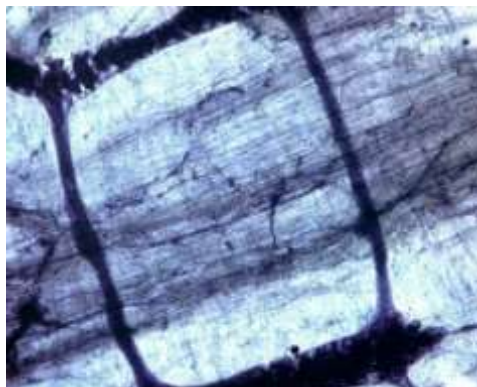


Fig 1.- Myenteric plexus. Guinea pig (Loevit)



### **E.7 DEMYELINATION OF CENTRAL TRACTS ASSOCIATED TO ARSENIC EXPOSURE IS ACCOMPANIED BY INCREASED PHOSPHORYLATED NEUROFILAMENTS IN RATS.**

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Demyelination and axonal degeneration in the central nervous system (CNS) are associated with mitochondrial alterations and anomalies of the neuronal cytoskeleton such as turnover and phosphorylation of its proteins. These signs of neuronal damage are present either in diseases caused by genetic defects or infectious agents, in autoimmune processes, in trauma and as a result of exposure to toxic agents. An animal model of progressive central demyelination is chronic arsenic exposure in rats, which is associated with behavioral deficits; also, exposed humans present cognitive deficits upon chronic arsenic exposure during childhood and puberty. In this model, we studied whether demyelination and axonal damage in the CNS is associated with alterations in complex IV mitochondrial and axonal cytoskeleton proteins such as neurofilaments. Using histochemical and immunohistochemical techniques we analyzed brain regions involved in memory, learning, motor and sensory functions, such as the hippocampus, cortex and striatum. Couples of Wistar rats received drinking water containing 3ppm of arsenic (approximate doses 0.4 mg/kg/day). After 10 days, males were retired and arsenic exposure continued in the pregnant rats throughout gestation and lactation. Offspring were weaned at 4 weeks and continued under arsenic exposure. At the age of 4 months the animals were anesthetized, transcardially perfused and the brains extracted and fixed. Seven micrometer sections were collected in silanized slides. Phosphorylated and non-phosphorylated neurofilaments were marked by immunohistochemistry, and the mitochondrial activity of cytochrome oxidase was assessed through histochemistry. The semi-quantitative data of this study demonstrated that demyelination in the striatum of exposed animals was accompanied by an increase of phosphorylated neurofilament immunoreactivity ( $p < 0.01$ ), while in the hippocampus of exposed animals, morphological alterations such increased volume of cell bodies, hyperchromasia and variations in cell structure were observed. These cellular changes were accompanied by an increase of phosphorylated neurofilament immunoreactivity ( $p < 0.001$ ). The results of the histological evaluation of the cortex showed no significant difference between the experimental groups. No differences were found on the histochemical staining of cytochrome oxidase in the 3 studied areas. These findings suggest that arsenic disrupts the neuronal cytoskeleton in regions involved in integration of sensory and motor information, at a level of exposure reported to disrupt learning in animal models.

## **E.8 SKELETAL MUSCLE HISTOLOGY IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS**

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Multiple sclerosis is a chronic neurodegenerative disease. Patients suffer motor impairment in many cases and there are a 65% of patients that have significant fatigue. The physiopathology of fatigue is complex, having identified both central and peripheral mechanisms. Among peripheral components, reduced oxidative metabolism in muscles and changes in fiber pattern have been reported.

There is an animal model, experimental autoimmune encephalomyelitis, that mimics the multiple sclerosis crisis suffered by patients; moreover, these animals show important motor impairment. The purpose of our study was to histologically characterize the skeletal muscle changes of animals affected by experimental autoimmune encephalomyelitis.

12 Dark Agouti rats were organized in three groups: normal control (any intervention), vehicle control (Freund's adjuvant) and experimental autoimmune encephalomyelitis (induction with MOG + Freund's adjuvant). Animals were sacrificed at day 38 postinduction and soleus muscles were removed. Both electron microscopy and light microscopy samples were taken. Histological and histochemical stains were performed on cryostat cuts. ImagePro Plus 4.5 was employed for morphometric analysis and SigmaStat 3.1 for statistical analysis.

Clinically, experimental autoimmune encephalomyelitis animals showed limbs and tail paresia that started on day 13 p.i. and got worse during the following days. Histologically, atrophied fibers with angulated profiles and occasional internal nuclei were observed. No necrosis or regeneration was noticed.

A cross-sectional area reduction and a decrease in the minor diameter were detected. Additionally, muscle fibers presented core-targetoid cytoarchitectural changes and it should be highlighted the appearance of intermediate fibers with ATPase pH 9.8. All these evidences point to a neurogenic muscular lesion in this experimental model.

Alterations in number and morphology of muscle mitochondria were also noticeable in electron microscopy.

Our data show that muscular impairment in this experimental model seems to mimic the histological characteristics of skeletal muscle in multiple sclerosis. Therefore, this is a valid model to study therapeutical strategies through the evaluation of muscle recovery.

## E.9 HISTOLOGY OF EXTRAOCULAR MUSCLES IN STRABISM

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Extraocular muscles (EOM) have a number of morphological and functional features that distinguish them from nonocular skeletal muscles. Normal EOM myofibers characteristics are so unique that they can not be included in the traditional skeletal muscle fiber classification. Moreover, they continue to express immature isoforms of the myosin heavy chain, immature form of the acetylchoine receptor and a number of growth factors and their receptors, normally downregulated in adult skeletal muscle. Moreover, it has been shown a continuous myonuclear addition (satellite cells proliferation and fusion) and removal (apoptosis) in individual myofibers troughout life. This can explain the preferential involvement or sparing of these muscles in myopathic and neurogenic disease.

For these reasons the study of EOM in pathologic situations can be useful to analyse skeletal muscle proplasic processes (renovation, regeneration and reparation) from a different point of view. In this study, our goal was the microscopic characterization of EOM in patients with strabism.

Human EOM muscles were obtained from patients undergoing surgical correction of strabism. Muscles were frozen in methylbutane cooled in liquid nitrogen and sectioned serially at 6  $\mu\text{m}$  on a cryostat. Histological stains (hematoxilin-eosin, Gomori's modified trichrome, van Giesson trichrome and Masson trichrome), histochemical techniques both non enzymatic (PAS, Oil Red 0, Alizarin Red, Acridine Orange) and enzymatic (NADH-tr, SDH, ATPasa, acid phosphatase, alkaline phosphatase), and immunohistochemical methods (myosin, desmin, Pax7) were performed.

Based on histological, histochemical and immunohistochemical analysis we classified the observed features in degenerative, regenerative and/or adaptative. However, it was difficult to determine if these changes were cause or consequence of strabism.

The microscopic study of EOM in strabism could be a valuable method for the study of proplasic processes in these unique muscles.

## **E.10 IDENTIFICATION OF A NEW HISTOCHEMICAL MARKER OF PERIPHERAL NERVE REGENERATION**

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Several cellular and molecular processes occur during peripheral nerve regeneration. Probably, the most accurate marker of regeneration is the identification of axonal sprouting with the formation of bands of Büngner. GAP-43 was initially described as a protein associated to cell growth, although its function is not well understood. In this work, we analyzed the expression of GAP-43 protein during nerve regeneration in a model of peripheral nerve injury.

A model of sciatic nerve defect was developed by generating a 10-mm nerve gap in Wistar rats. Then, the nerve gap was repaired using a NeuraGen® conduit consisting of lyophilized type I collagen and the regeneration process was evaluated after 12 weeks. Tissues corresponding to the damaged sciatic nerve were fixed in formalin and embedded in paraffin for histological analysis and immunohistochemistry for GAP-43 and neurofilament protein expression.

GAP-43 and neurofilament were expressed in regenerating nerve fascicles, although each protein was associated to a different structure. On one hand, GAP-43 was positive in some thin-axons of the regenerated fascicles with an axoplasmatic pattern. On the other hand, NFL was positive in thick-axons of the regenerated fascicles with a cytoplasmic pattern.

These results reveal that GAP-43 is a marker of peripheral nerve regeneration. The fact that GAP-43 expression was not associated to neurofilament, suggests that GAP-43 could be a marker of initial collateral sprouting. In conclusion, both NFL and GAP-43 should be used to carry out a complete assessment of the axonal regeneration process.

This work was supported by grant IPT-2011-0742-900000 from the Spanish Ministry of Science and Innovation (co-financed by FEDER).

## E.11 METHYLENE BLUE APPLICATION AS A THERAPEUTIC STRATEGY TO TREAT EXPERIMENTAL ROP

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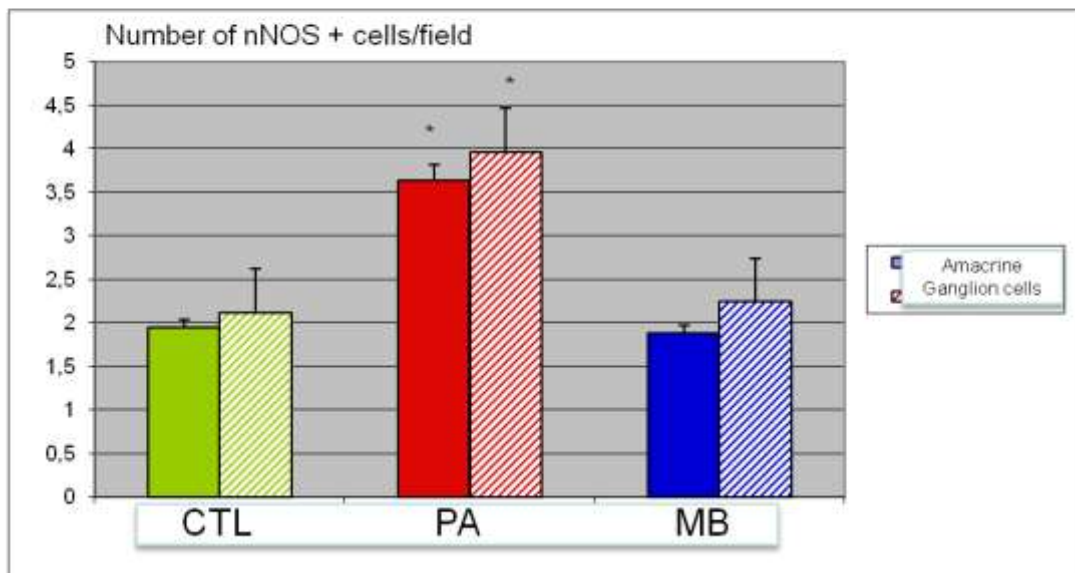
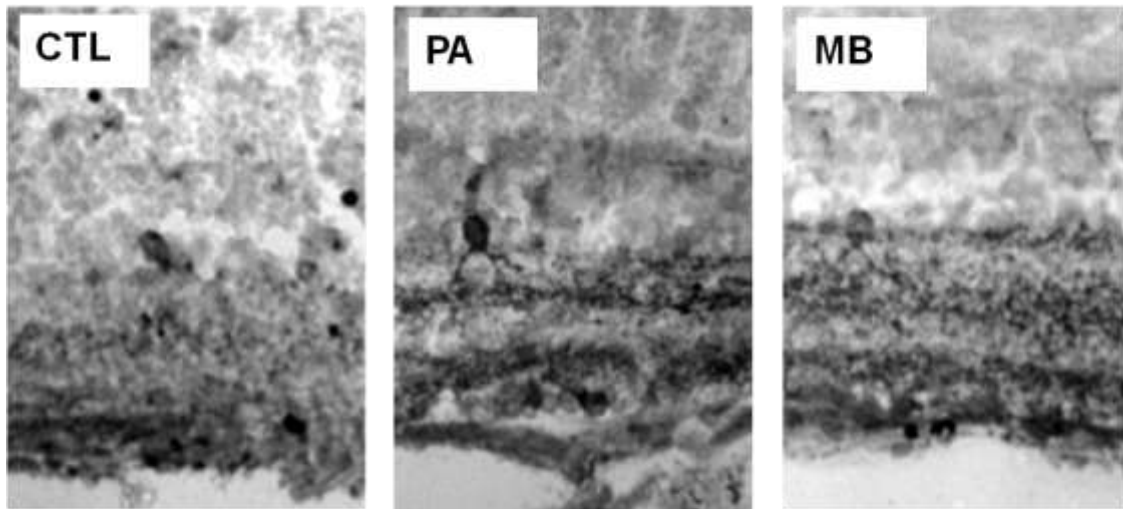
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Perinatal asphyxia is able to induce retinal lesions, generating ischemic proliferative retinopathy (IPR) resulting, in severe cases, in blindness. Previously we have demonstrated in this model retinal neurodegeneration, gliosis, and neovascularization, which are compatible with retinopathy of prematurity (ROP). Furthermore we have identified the involvement of the nitrergic system in this physiopathology. We hypothesize the participation of nitric oxide (NO) through the neuronal isoform of the enzyme nitric oxide synthase (nNOS) as trigger of the previously observed structural and molecular alterations, and analyze the application of methylene blue (MB), a NOS inhibitor, as a therapeutic strategy. We used 30 days-old male Sprague-Dawley rats (n=5/group) obtained as follows: 1) PA were animals exposed to perinatal asphyxia (20 min., at 37°C), 2) PA+MB were animals born from pregnant to term female rats treated with MB (2 mg/kg) 30 and 5 min before delivery and subjected to PA induction during 20 min. at 37°C, 3) CTL were born to term animals. Constitutive NOS enzymatic activity, NADPH diaphorase histochemical method, immunohistochemistry and Western-blot assay for nNOS were used to evaluate retinas. A significant increase of nNOS activity was observed in retinas of 30 day-old animals subjected to PA compared to CTL (PA=10,8±0,4; CTL=9,1±0,3 pmol/min/mg protein, p<0,05), while PA+MB animals showed no significant differences with CTL. This was correlated with a 28% significant increment in nNOS expression in PA vs CTL with a significant 60% reduction in PA+MB vs CTL. Histochemical and immunohistochemical studies support these results, with nNOS localization in ganglionar and amacrine cells. Application of MB as a therapeutic strategy showed a strong effect inhibiting constitutive NOS activity and nNOS expression. This finding stimulates future studies aiming to use MB as a new treatment to avoid or decrease retinal damage in the context of ROP.

## nNOS Immunohistochemistry



## **E.12 ULTRASTRUCTURAL CHANGES IN THE PROGRESS OF NATURAL SCRAPIE**

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Universidad de Zaragoza

Transmissible Spongiform Encephalopathies (TSEs) are a group of neurodegenerative diseases which affect animals and human beings with unavoidably lethal end. The most accepted theory about the causal agent is based on an abnormal isoform (pathological prion protein, PrP<sup>sc</sup>) of a cellular protein (PrP<sup>c</sup>) physiologically located in nervous tissue. Spongiform changes and PrP<sup>sc</sup> accumulation are the main features of this pathology. By electron microscopic, SAF (Scrapie associated fibrils) observation has been confirmed as the pathognomonic sign in TSEs. However, studies about the ultrastructural pathological changes of the disease have been scarcely developed. Besides the vacuolization, tubulovesicular structures, coated spiral membranes and pits and membrane proliferation have been described, but with contradictory results in some occasions.

The main aim of the present study consisted of consistently determining specific lesions for this group of diseases at ultrastructural level. To demonstrate whether these changes were evident regardless the stage of the disease was also intended in order to help to clarify the lesional progress of this neurodegenerative disease.

All and each one of the pathological changes cited by other authors in relation with TSEs was assessed by electron microscopy in sections of cerebellum from natural Scrapie cases in comparison with control healthy cases. They were all found, although in low frequency and not in all samples suggesting a great difficulty to recognize them and a consequent low representative rate.

Otherwise, a close relationship between astrocytes and vacuolation of Purkinje cells was constantly evidenced in all cerebella studied. Therefore, a relevant role of astrocytes in the progress of the lesions seems to be demonstrated here.

**E13. PERIPHERAL NERVE RECONSTRUCTION WITH CONDUITS SEEDED WITH VASOACTIVE INTESTINAL PEPTIDE GENE-TRANSFECTED MESENCHYMAL STEM CELLS IN A RAT MODEL.**

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Attempts have been made to improve peripheral nerve reconstruction. Vasoactive intestinal peptide (VIP) is a neuropeptide with neuroprotective actions, trophic and regulatory development.

We studied a unilateral peripheral nerve injury repair model with 10-mm injury using DL-lactic-ε-caprolactone conduits in rat sciatic nerve in order to evaluate the potential therapeutic usefulness of the VIP neuropeptide in peripheral nerve injury.

Twenty-four male Wistar rats were distributed into four groups: NI group, nerve injury with no repair; CC group, nerve injury repaired with conduits; CC-VIP group; nerve injury repaired with conduits plus local VIP instillation; CC-ASC-VIP, nerve injury repaired with conduits seeded with adipose-derived mesenchymal stem cells transduced with VIP-expressing lentivirus. We assessed limb function by measuring ankle stance angle and percentage muscle mass reduction and evaluated the histopathology and morphometry of myelinated fibers.

After 12 weeks, the best angle of ankle position was observed in the group CC-ASC [ $40.32 \pm 3.68^\circ$  in the NI group,  $40.80 \pm 11.27^\circ$  in the CC group,  $44.12 \pm 5.26^\circ$  in the CC-VIP group and  $52.61 \pm 4.04^\circ$  in the CC-ASC group (Kruskal Wallis,  $p = 0.04$ )], as the highest myelinated fibers found in the operated distal nerve segment [CC-ASC:  $9596.16 \pm 4808.74$  fibers / mm<sup>2</sup>, CCVIP:  $6647.04 \pm 3239.96$  fibers / mm<sup>2</sup>, DC:  $3720.96 \pm 1368.66$  fibers / mm<sup>2</sup>, NI:  $483.84 \pm 838.03$  fibers/mm<sup>2</sup> (Kruskal Wallis,  $p = 0.042$ )].

Our results suggest that a cell substrate with a source of VIP could be a promising tool for improving guided nerve regeneration through ducts of DL-lactide-ε-caprolactone and therefore be of potential value in future translational repair of peripheral nerve injuries in the clinic.



#### **E.14 GIANT AXONAL NEUROPATHY (VARIANT OF HEREDITARY MOTOR-SENSORY NEUROPATHIES).**

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Giant Axonal Neuropathy (GAN) is a rare disease named for the enormous enlargement of the peripheral nerve axons due to excessive formation of neurofilaments. It was first described in a 6-year-old with a gradually progressive motor-sensory neuropathy that began at 3 years of age (Asbury, 1972). Subsequent case reports have established that this disease is an entity that is only seen in children with clinical features that include progressive distal symmetric polyneuropathy, and ataxia, usually accompanied by a bilateral extension plantar response with an abnormal EEG and in some cases without mental retardation. The individual can also present curly or kinky hair. GAN has an autosomal recessive inheritance pattern. The age of onset is 2 to 5 years with a clumsy gait and a tendency to fall. The mutations are located in the gigaxonin (GAN) gene on chromosome 16q24.1.

The patient is a 3.5 year-old female. Her disease began at 14 months when her development stopped. She had difficulty getting up and could not walk; ability she achieved with difficulty at 18 months with incoordination. Her gait is currently independent and she is able to perform tandem gait. Her muscle strength is 4/5 in the lower limbs, sensitivity is preserved, but she cannot maintain orthostatic balance, she has bilateral lateropulsion and her muscle reflex responses are decreased. NCSs are abnormal with involvement of the four limbs, which is suggestive of Hereditary Motor-Sensory Neuropathy.

The Sural Nerve biopsy was fixed in 2.5% glutaraldehyde buffered and divided. One fragment was processed for paraffin embedding (using routine and special staining methods and immunostaining with anti-neurofilaments and anti-myelin). A second portion was used for inclusion in epoxy resin and analysis by both high-resolution optical microscopy and transmission electron microscopy. A decrease of mainly medium diameter myelinated axons were observed, in addition to very extraordinary large axons almost three times the average intermediate diameter with dimensions ranging from 11 to 25

microns. (See Fig. 4) in which huge clusters of neurofilaments that distend the axoplasm and significantly thin the myelin sheath are found in their paranodal segments. There are scarce fibers with atrophic, dystrophic or degenerative changes, characterized by axoplasmic retraction, clusters of membranous vesicles, osmiophilic material deposits, and changes in myelin sheath structure (swelling, disruption or delamination, and thickness reduction).

Clinical data and structural findings, mainly observed in the biopsy, can determine this case as a variant of Hereditary Motor-Sensory Neuropathy called "Giant Axonal Neuropathy."

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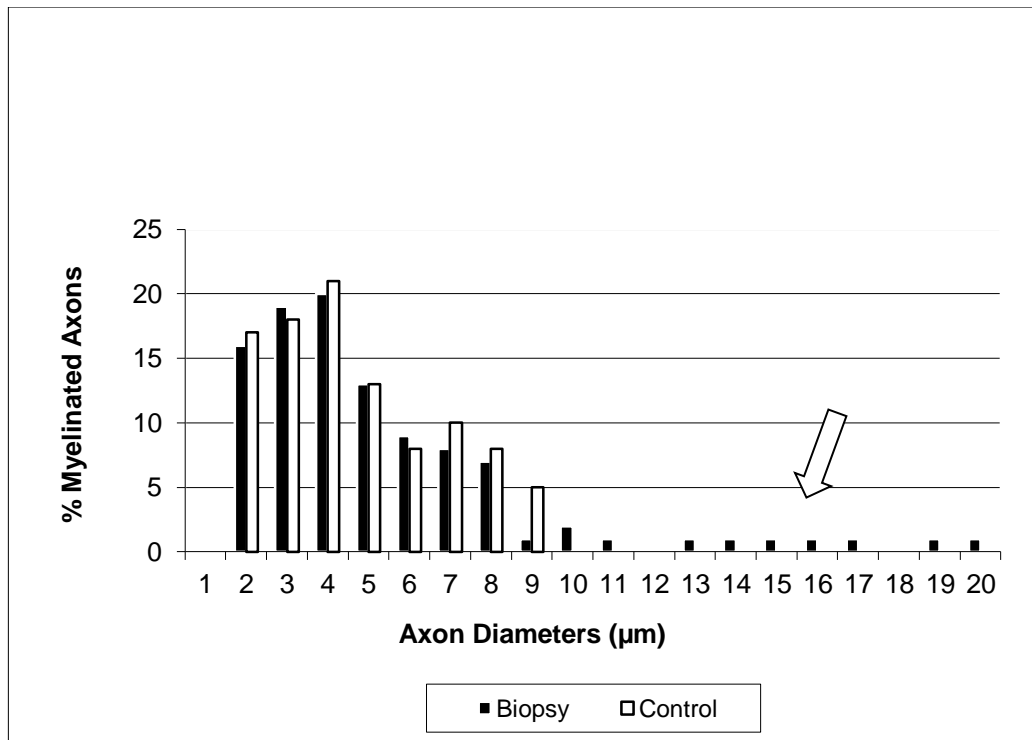


Figure 4. Histogram of the sural nerve, which shows the presence of large myelinated axons (arrow) with abnormalities in distribution and relative to the normal pattern.

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## F. Histological Techniques

### F.1 IMMUNOHISTOLOGY: “A FANTASTIC VOYAGE” THROUGH THE NORMAL HUMAN BODY

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Immunohistochemistry is a technology that has revolutionized the scientific scene in Cellular Biology and Histology since its beginnings as it allows the *in situ* identification of any protein while respecting the morphologic substrate. Immunohistochemistry was developed from the immunofluorescent technique and it is fair to say, that no special technique has influenced the way that Pathology is practiced so profoundly as immunohistochemistry. In fact, the spectacular development that immunohistochemistry has been living over the last decades is intimately connected to its enormous usefulness in the histopathologic diagnosis. Consequently, the number of available antibodies for paraffin sections is constantly increasing and the quality of visualization systems is improving so that today it is possible to demonstrate even the least represented antigens.

Furthermore, both immunohistochemistry and immunofluorescence make the labelling of multiple antigens in the same histological section possible, which allows us to clarify if the different molecules are colocalized in the same cell or in different tissue compartments. Multiple labelling is particularly valuable for us, the histologists, as it permits the analysis of cellular populations as complex as those that integrate the lymphatic tissues, the hypophysis, the pancreatic islands or the epidermis. Immunohistochemistry may also be combined with different classical staining methods used in Histology, such as the techniques to demonstrate carbohydrates, which allows us to obtain histologic images of incredible beauty.

The main objective of the lecture will be to undertake a kind of “fantastic voyage” through the different tissues and organs of the normal human body “labelled” by immunohistochemistry. As a consequence, we could check its usefulness in teaching Histology by several examples, such as the easy identification of the different visceral layers, or the localization of the different cell populations that integrate every tissue, organ or system in the context of a healthy individual. Our aim is to substitute the classical concept of a pure “morphological histology” by the new one of “molecular histology”, the Immunohistology, in order to prepare our students to have a better understanding of the pathological state.

## **F. 2 4D-MICROSCOPY TO STUDY DEVELOPMENT AND APOPTOSIS IN *C. ELEGANS***

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During development, the processes of cell division, differentiation and apoptosis must be precisely coordinated in order to maintain tissue homeostasis. The nematode *Caenorhabditis elegans* is a powerful model system to study development and cell death. It is small, transparent for ease of manipulation and observation. What is unique to this organism is that wild-type individuals contain a constant 959 cells and the position of cells is constant as is the cell number. The analysis of the lineage of an embryo using a 4D-microscope provides a clear picture of the origin and fate of each individual cell, as well as the position and migration in the embryo. During development of *C. elegans*, cell death occurs in specific cells of its invariant lineage. That apoptotic cells condense and form refractile corpses very easy to visualize under differential interference contrast (DIC) microscopy. Cell division, differentiation and apoptosis is precisely coordinated and the apoptotic corpses in embryos are engulfed and digested by neighbouring cells. These characteristics has made that in recent years, the nematode *C. elegans*, has become an important in vivo model for the study of development and cell death.

### **F. 3 COMPARATIVE STUDY ON MASSON'S TRICHROME AND PICROSIRIUS RED TECHNIQUES FOR COLLAGEN HISTOPATHOLOGICAL CHARACTERISATION**

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Owing to the wide diversity of biological processes and structures in which collagen remodelling intervenes, collagen characterisation in histopathological assessments requires a valid, reliable and objective measuring technique. In this study we compared the routine Masson's trichrome and picrosirius red stainings in terms of collagen bundle orientation and occupied area.

We obtained 48 autopsic skin samples in which Masson's trichrome and picrosirius red stainings were performed. In order to examine the collagen fibers, five pictures with a 200x magnification of the reticular dermis were obtained for each case of each staining, using clear field microscopy and polarized light respectively. They were processed using the image analyzer software Image-Pro Plus 7.0. The area filled with collagen and the collagen bundle orientation by Fourier analysis were measured twice by two different observers in every picture and the mean of all five pictures was used to obtain the mean of each case. The data obtained underwent a statistical analysis with SPSS 17.0 software. A p-value < 0.05 was considered significant.

The measurements of the collagen bundle orientation and the area filled with collagen in Masson's trichrome and picrosirius red techniques were strongly correlated. This correlation was demonstrated after performing both the consistency and the absolute agreement intraclass correlation coefficient as well as the Cronbach's alpha coefficient. The measurements obtained by Fourier analysis of the collagen bundle organization in the samples stained with picrosirius red by two observers showed an almost perfect agreement (ICC>0.8). Following the same line, the results given in the Masson's trichrome group by both observers for the collagen orientation surpassed an ICC>0.8 as well. The same statistical procedure was applied in the case of the area filled with collagen, being significantly concurrent only in the staining with Masson's trichrome.

Some authors advocate the picrosirius red as preferred procedure for isolating collagen fibers of other artefacts stained in the trichrome such as nuclei and the extracellular matrix. Nevertheless both staining methods provide valid data for histopathologic evaluation of collagen fibers, excluding these artefacts as a source of skewness.

It has been proven in this study that Masson's trichrome is a reliable method for collagen characterisation. However, the interobserver variability shown in picrosirius red regarding the collagen area parameter makes the trichrome an election technique.

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Noorlander ML, Melis P, Jonker A, Van noorden CJ. A quantitative method to determine the orientation of collagen fibers in the dermis. J Histochem Cytochem. 2002;50(11):1469-74.

<b>Correlation between picosirius red and Masson's trichrome measures</b>		
<i>Orientation</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,853	0,856	0,921
<i>Area filled with collagen</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,732	0,729	0,825
<b>Test reliability: Interobserver variability within Masson's trichrome</b>		
<i>Orientation</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,91	0,903	0,953
<i>Area filled with collagen</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,833	0,516	0,909
<b>Test reliability: Interobserver variability within picosirius red</b>		
<i>Orientation</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,94	0,928	0,969
<i>Area filled with collagen</i>		
Consistency ICC	Absolute agreement ICC	Cronbach's alpha
0,546	0,491	0,706

**Figure 1**

#### F.4 CHARACTERIZATION OF THE DIFFERENTIATION PROCESS IN THE HUMAN FUNDIC GLANDS BY SIALIC ACID-BINDING LECTINS

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The fundic unit of the human stomach is formed by the gastric pit and the fundic gland, and contains 5 major mature types of epithelial cells: surface mucous cells (or gastric pit mucous cells), parietal cells, mucous neck cells, chief cells, and endocrine cells. The stem cells are located at the union between the gastric pit and the isthmus of the gland. The chief cells are located at the bottom of the gland. For many time the origin of the chief cells was unknown because no mitosis were observed at the bottom of the gland and because they were far from the isthmus, where mitosis were observed. Since some years ago growing evidences suggest that there is a differentiation route: mucous neck cells – pre-zymogen (pre-chief, transitional) cells – chief (zymogen) cells [1]. However, this is not an easy process because mucous neck cells and chief cells are very different cells secreting very different substances. Our aim was the use of sialic acid-bind lectins to study this differentiation process at electron microscopy level.

Six healthy samples were obtained from the fundus region of human stomachs. The samples were fixed in 10% formalin in PBS for light microscopy, or in 2% glutaraldehyde for electron microscopy. Then, they were embedded in paraffin or in Lowicryl K4M, respectively. Sections were obtained and incubated with digoxigenin labeled *Sambucus nigra* lectin (SNA) and then with anti-digoxigenin antibodies. These antibodies were labeled to peroxidase (HRP) for light microscopy, or to colloidal gold for electron microscopy. HRP was developed with 3,3'-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub>. SNA labels to the terminal sialic acid  $\alpha(2-6)$ -linked. *Maackia amurensis* lectin (MAA), which labels to sialic acid  $\alpha(2-3)$ -linked, and *Limax flavus* lectin (LFA), which labels to sialic acid, were also used at light microscopy level [2]. Wheat germ lectin (WGA), which recognizes GlcNAc and sialic acid [2], were also used at light microscopy, with and without sample pre-digestion with neuraminidase.

At light microscopic level, MAA was negative, while WGA was positive, but this staining remained after neuraminidase predigestion suggesting that the presence of GlcNAc. LFA showed a similar but lighter staining than SNA. So, SNA was used for the electron microscopy study.

With SNA at electron microscopy level, the surface and gastric pit mucous cells were unstained. The mucous neck cells showed biphasic granules, showing a small and round electrondense core within an electronlucent granule. The lectin slightly labeled the electronlucent regions of the granules and the trans-Golgi region. The transitional or pre-zymogen cells showed electronlucent secretory granules with electrondense regions, which increase in size as the cell position descends in the gland. The transitional cells showed a similar binding pattern to that of the mucous neck cells (Fig 1). The chief cells showed large electrondense secretory granules scarcely labeled. The parietal cells were labeled in the plasmatic membrane of the intracellular canaliculi. Some immature parietal cells were observed, showing electronlucent granules positive to the lectin.

In conclusion, the presence of oligosaccharides with terminal sialic acid  $\alpha(2-6)$ -linked has been detected in the electronlucent region of the granules of the mucous neck cells and transitional cells, while the electrondense regions and the electrondense granules of the chief

cells were negative. This suggests that during the differentiation process from mucous neck cells to chief cells, the content of the granules is progressively modified.

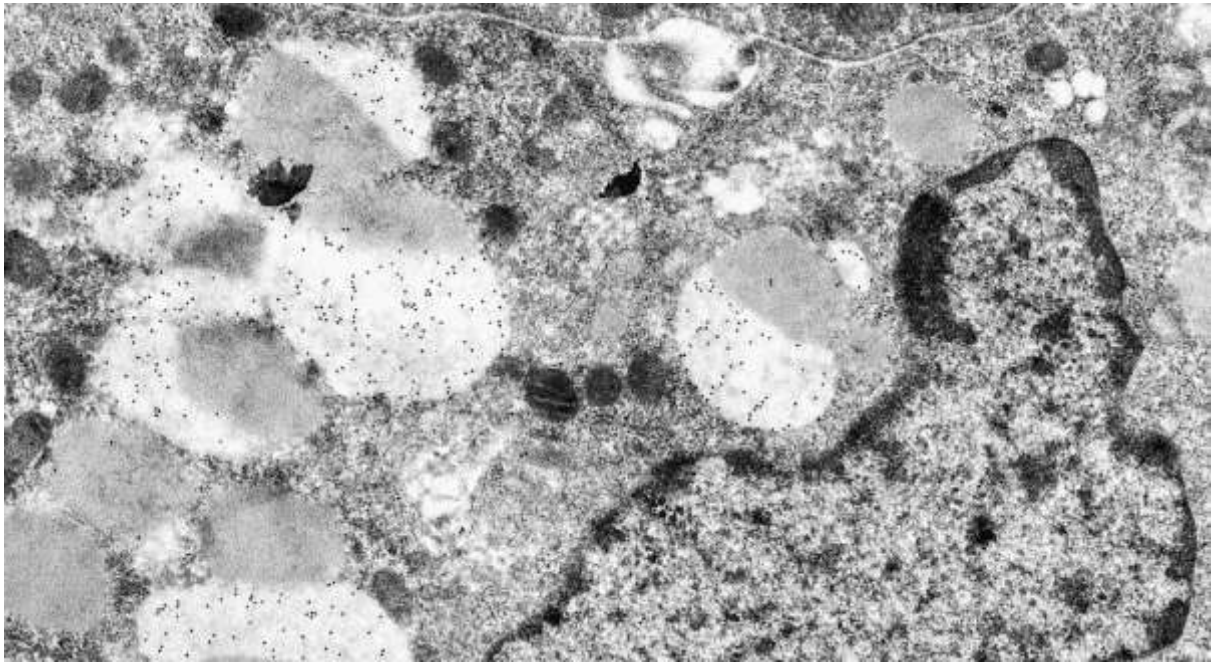


Figure 1. Transitional cell. The secretory granules are biphasic showing electron-dense and electron-lucent regions. Note the lectin labeling over the electron-lucent regions.

W. Hoffmann. *Front Biosci.* 5, 720-731 (2013)

N. Shibuya, I.J. Goldstein, W.F. Broeckaert, M. Nsimba-Lubaki, B. Peeters and W.J. Pneumans. *J. Biol Chem* 262, 1596-1601 (1987)



## **F.5 BIOPSY FOAM PADS ARTIFACTS CAN MIMIC CHOLESTEROL CRYSTAL EMBOLISM**

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Cholesterol crystal embolism is an infrequent disease that can affect kidney, muscles and other organs. Atherosclerotic plaques can break off and cholesterol crystals are liberated into the arterial circulation, then are deposited in small arteries and arterioles and are histologically seen as empty spiculated spaces inside these vessels.

Biopsy foam pads are inserted into plastic tissue cassettes to prevent the escape of small specimens, such as core needle biopsies, and to maintain the tissue on a flat surface to facilitate the paraffin block construction and posterior sectioning. These foam pads can create a mark on tissues, especially if it's fresh or partially fixed, producing "atheroembolia-like" images.

To establish if the use of biopsy sponges during the processing of core needle biopsies can create fake images of atheroembolia.

Needle core biopsies from surgical and autopsic specimens were performed. The small cylinders of tissue from core needle biopsies were processed in two different ways:

Tissue in direct contact with the biopsy foam pad.

Using paper wraps between the tissue and the foam pad.

In the biopsies processed in direct contact with the biopsy foam pad many histological artifacts, in the form of empty spiculated spaces, were found. These images could be mistaken as intra-arterial cholesterol crystals.

These artifacts weren't spotted on the biopsies processed with paper wraps between the tissue and the biopsy sponge.

Core needle biopsies processed in direct contact with the biopsy foam pad can produce histological artifacts that can be misinterpreted as atheroembolia.

The incidence of these artifacts can be reduced if the tissue is completely fixed before the use of the foam pad, or by using paper wraps between the tissue and the foam pad.

## **F.6 SELECTIVE IRON DEPOSITS IN THE GONADOTROPIC CELLS OF ADENOHYPOPHYSIS ARE THE CAUSE OF HYPOGONADISM IN HEMOCHROMATOSIS**

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Hemochromatosis is an inherited disease produced by an iron overload leading to their accumulation in some parenchyma that produces morphological alterations in different organs, causing their malfunction. Clinically it can manifest, among other symptoms, with cirrhosis of the liver, diabetes mellitus and hypogonadism.

Iron deposits can be seen histologically in the liver or pancreatic parenchyma. However, no significant iron deposits are detected in the testicular parenchyma, even though the apparent germ cells atrophy. Testicular function is regulated by the hypothalamic–pituitary–gonadal axis. When the adenohypophysis (anterior pituitary gland) is studied, no significant iron deposits are seen that could justify its dysfunction.

To assess if iron deposits in adenohypophysis are limited to a specific cell type

Immunohistochemical stains were performed in order to detect pituitary hormones (FSH, LH, ACTH, prolactin, GH and TSH) on paraffin sections of a formalin-fixed pituitary gland from a patient with hemochromatosis. At the end of the protocol a Perls technique for iron staining was performed instead of the standard hematoxylin counterstaining.

Most of the iron deposits were observed in the cells that expressed FSH or LH. The others pituitary cells, which expressed the rest of hormones, almost didn't show any iron deposit.

The hypogonadism in hemochromatosis seems to be caused by the selective iron deposits into the cells that produce gonadotropic hormones (LH and FSH) in the pituitary gland.

## **F.7 AUTOMATIC CLASSIFICATION OF RETICULIN FIBERS PATTERNS BASED ON A DICTIONARY LEARNING STRATEGY.**

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The analysis and classification of reticulin-fiber patterns is a valuable tool in some neoplasms. In order to help the characterization of the reticulin fiber network in neuroblastoma, an application to automatically classify reticulin-fiber patterns from biopsy images has been designed and implemented.

We used a dataset consisting on 80 images obtained from Gomori staining in tumor tissue biopsies from different patients. Each image was manually classified according to its main reticulin-fiber pattern as organoid/reticular or lobular/trabecular, according to similar distributions in different human organs. The reticulin pattern classification model involves a local dictionary training based on an input image, which is compared to a global dictionary to obtain an input signature or array of correlations. The dimensionality of the signature is reduced by removing the less discriminative features according to the ReliefF scores obtained. Comparing the reduced signature with a signature set previously calculated from the global dictionary we are able to apply a Knn supervised classifier to assign a pattern class to the input image. The dictionaries used in this work are generated using the KSVD algorithm, a sparse representation technique which guaranties the maximum representation in a dictionary with a limited number of atoms. A list of selected feature indexes from the reduction of the signature set is saved to process the input images in the classification pipeline as shown in the figure.

The performance of the model was tested by a leave-one-out cross validation strategy which showed an accuracy of 64.56% and a balanced accuracy rate of 55%. Other patterns are being identified based on clinic-biological features of the tumors.

The automated classification of image-based reticulin fiber patterns is necessary to analyze huge amounts of images, which may be useful in detecting and delineating tumor and stromal cell interactions.

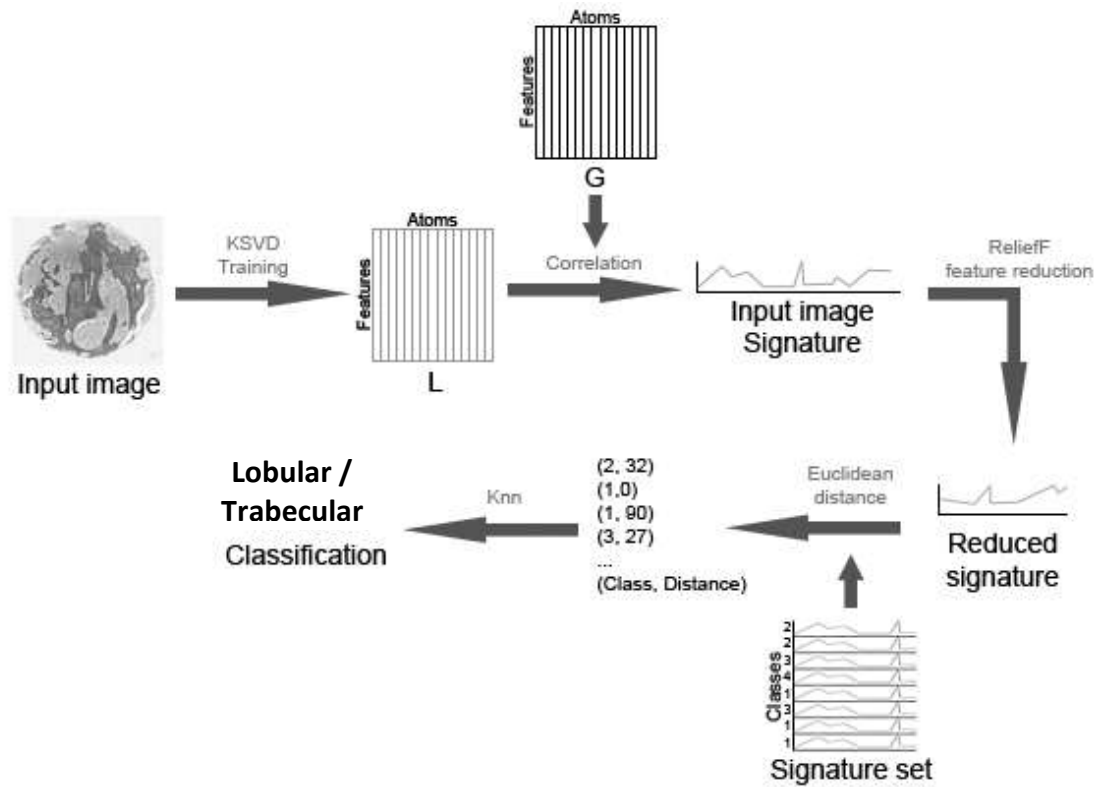


Figure 1: Classification pipeline showing the steps to classify an input image. L: Local dictionary; G: Global dictionary.

Grants: ISCIII2012-RED-36-020, ISCIII2010-PI00015.

## **F.8 MORPHOMETRY OF SKELETAL MUSCLE FIBERS: IMMUNOHISTOCHEMICAL AND IMAGE ANALYSIS**

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In an experimental study to assess anti atrophy treatment of muscle fibers (type I slow) we developed a protocol to measure some morphometric parameters of the muscle fibers in each case.

The study was carried out with mice hindlimb-unloading for 14 days, with or without treatment and compared with a control group treated or not. The soleus muscles were embedded in paraffin for cutting perpendicular to the midpoint of the muscle and stained with reticulin (Gomori) and by immunohistochemistry anti-MHC I to detect type I muscle fibers. Serially ordered pictures of the soleus cross section (10-14 pictures of 2048 x 1536 pixels at 20x) were mounted to obtain a single image of full muscle section for each case among 22-38 Megapixels at 20x that was digitally analyzed.

Reticulin dyed inside the fibers lighter than the sheath that surrounds (endomysium) that was stained dark, allowing us to separate and binarize (segmentation) simply and extremely accurate. In the immunohistochemical staining the segmentation and separation was poorer so we moved the positively stained fibers to the binary mask obtained in reticulin staining of the same sample, which as we said is much more accurate. For each case and stain we measured area, minimum diameter, perimeter, and roundness of the muscle fibers and the area, major and minor axis of full soleus.

This morphometric protocol, combining histochemistry and image analysis based in reticulin stain provides highly accurate results in the measurement of skeletal muscle fibers and efficiently because 900-1000 fibers were measured by case.

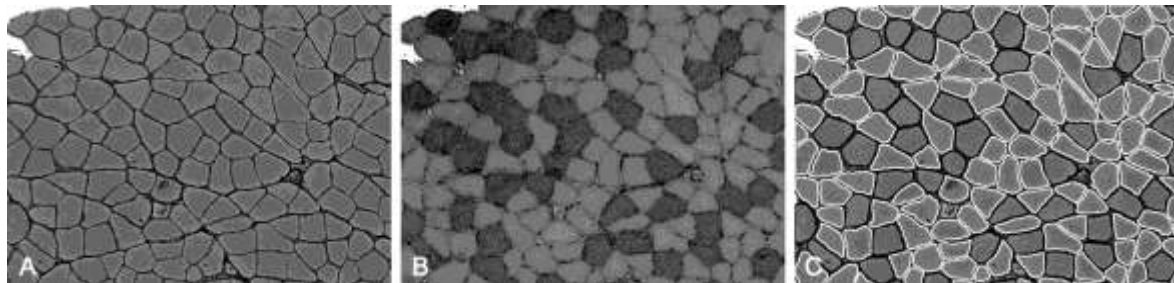


Fig1. Detail of muscle fibers at 20x: A) Reticulin. B) Immunohistochemistry of Type I. C) Binarization over reticulin, Type I black, Type II white.

## **F.9 HISTOLOGY TECHNIQUES APPLIED TO THE STUDY OF THE ADHESION AND INTERACTION OF CELLS ONTO OPAQUE OR TRANSLUCENT MATERIALS**

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Tissue engineering strategies involve the study of the adhesion and interaction of culture cells on biocompatible materials. For this purpose histological techniques are applied, both considering a whole-mount approach, i.e. studying the cells distributed on the surface of the material or slicing the sample. The whole-mount analysis can yield some information that cannot be obtained, at least easily, by the histological study of sections of the sample. Furthermore, some of the materials are very hard and cannot be sliced using the habitual microtomy procedures. On the other hand, the opaque or translucent nature of some of the materials used in tissue engineering complicate the whole-mount sample observation by light microscopy.

In this communication we describe the procedure that we are developing to obtain the maximal histological information from whole-mount samples of cells cultured on opaque materials before any further embedding procedure.

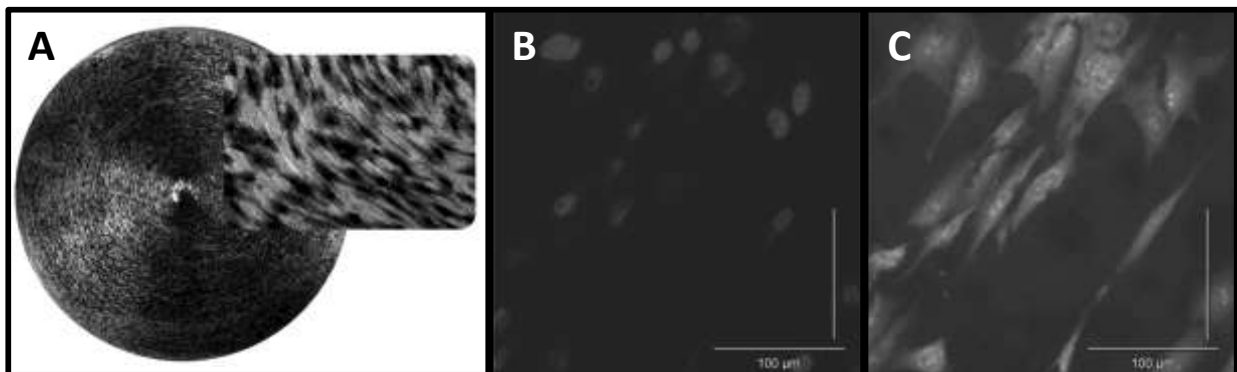
Dental pulp stem cells and MG-63 cell line were used. Cells were grown in 24-well plates, seeded on the selected biocompatible material (titanium discs, dentin slices, etc.) and samples were analyzed at several incubation time points. Cell culture media was removed and samples were fixed in 2.5% glutaraldehyde for 30 min and sequentially stained with hematoxylin and eosin for 5 min and incubated with 30  $\mu$ M DAPI for 10 min. The material was placed on a slide and observed using the appropriate filters on a fluorescence microscope to which a digital camera system was connected. Images of randomly selected areas were recorded and analyzed using the morphometric analysis program Image-Pro Plus (MediaCybernetics). Some samples were further processed for scanning electron microscopy.

The opaque or translucent nature of the materials impeded the direct *in vivo* observation of cells growing on them. After staining the cells with hematoxylin or cresyl violet it was possible to observe them, both by using an inverted microscope (after placing the material upside down) or a direct microscope (figure, panel A). The stained cells could be distinguished from the material background, but it was difficult to carry out a morphometric study as the limits of the cells were somehow difficult to determine. The use of fluorescent dyes greatly improved the resolution and allowed to obtain images of each sample from which both cell density, based on the nuclei count after DAPI dye binding and cell morphology parameters, based on the cell components staining with eosin (figure, panel C) were calculated.

Subsequently the samples were processed for scanning electron microscope. The morphology of the cells of these samples was similar to that of cells from samples that were not labeled with DAPI and eosin.

We describe a simple procedure that allowed us to obtain maximal histological data from each whole-mount sample of cells growing on opaque or translucent biocompatible materials. Thus, from light microscopic studies it was possible to determine cell density and morphological parameters. Furthermore, the sample could be later analyzed by scanning

electron microscopy. We are currently optimizing the method to diminish the interference of the fluorescence dyes binding to biological materials.



## F.10 A QUANTITATIVE CYTOCHEMICAL METHOD TO EVALUATE ELEMENT CONCENTRATIONS IN CELL CULTURE

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<sup>4</sup> Division of Traumatology and Orthopedic Surgery.

Ionic quantification of biological tissues depends on the availability of suitable standards. The concentration of an element in a biological sample is proportional to the net accounts characteristic signal of chemical element divided by the continuous radiation (peak/background) as determined in spectra obtained by electron-probe X-ray microanalysis using electron microscopy. The basic characteristics required for a compound to be used as a standard material are: well-defined chemical composition, homogeneity at the spatial resolution used during the analysis and assurance of similar interactions with the electron beam. This suitability can be determined by means of a calibration constant using the Peak/Background method (P/K). The objective of this work is to establish appropriate standards to quantify chemical elements within the cells using X-ray microanalytical cytochemistry.

Several inorganic salts (CL<sub>2</sub>Mg, PO<sub>4</sub>H<sub>2</sub>K, Cl<sub>2</sub>Ca y SO<sub>4</sub>Na<sub>2</sub>) were dissolved in 20% dextran at different concentrations. Small droplets of these solutions were deposited on pioloform-covered gold grids. Then, grids were immediately plunge-frozen in liquid nitrogen and freeze-dried. The elements contained in each salt were immediately analyzed using a scanning electron microscope with a microanalytical detection unit to prevent contamination or chemical modification. To determine the calibration constant, we calculated the coefficients of correlation (R) for increasing concentrations of each element.

Our results show that the range of coefficient of correlation was between 0.97 and 0.99 for all salts studied. Therefore, the salts analyzed in this work were appropriate to be used as standards for intracellular quantification. To determine the concentration of the major intracellular chemical element (Na, K, P, Ca, Mg, Cl and S), the calibration constant obtained with these salts can be applied to establish the intracellular ionic concentration of these element. Accordingly, the following formula can be used:  $C=K (P/B)$ , where C is the concentration of the standard, K the calibration constant and P/B is the Peak/Background ratio for each element.

This technology allows the quantitative analysis of cultured cells, and makes possible the in situ quantitative determination of the main intracellular elements that play a role in cell viability and cell function. This method could be used as part of the quality control process of cultured cells and tissues for clinical use.

Alaminos M, Sánchez-Quevedo MC, Muñoz-Ávila JI; García JM, Crespo PV, González-Andrades M, Campos A (2007). Evaluation of the Viability of Cultured Corneal Endothelial Cells by Quantitative Electron Probe X-Ray Microanalysis *Journal of Cellular Physiology*. 211(3):692-8.

Alaminos M, González-Andrades M, Muñoz-Ávila JI, Garzón I, Sánchez-Quevedo MC, Campos A. (2008). Volumetric and ionic regulation during the in vitro development of a corneal endothelial barrier. *Exp Eye Res*. 86(5):758-69.

This work was supported by grant SAS PI-0808-2010 from Consejería de Salud y Bienestar Social, Junta de Andalucía, Spain.



## F.11 A QUANTITATIVE MICROANALYTICAL STUDY TO EVALUATE CANCELLOUS BONE

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Adult cancellous bone consists of a series of trabeculae of mature lamellar bone. The lamellar bone tissue is hard, and it is considered as abiphasic tissue composed of a mineralized matrix and an organic material. The mineralized matrix component is hydroxyapatite. Scanning electron microscopy (SEM) associated to microanalytical detectors have been suggested as an excellent approach to obtain information on mineralized tissue. The objective of this work is to determine the concentration of calcium and phosphorus in mature lamellar bone corresponding to cancellous bone.

20 cancellous bone tissue biopsies were obtained from the femoral head of 10 patients with a diagnosis of femoral arthrosis and 10 patients with spontaneous fracture of the femoral head treated surgically. Each biopsy was fixed in liquid nitrogen and cryopreserved at -80°C. Then, specimens were washed in 3% H<sub>2</sub>O<sub>2</sub> for 24h to eliminate the organic component, air-dried, sputter-coated with carbon in an argon atmosphere (P=0.1 torr) and examined in a XL30 Philips scanning electron microscope (voltage= 15 kV; spot size = 50nm; tilt angle = 35°; take-off angle = 50°) with an EDAX microanalytical detector. Spectra were collected by pin-point electron beam at 40000X magnification. Twenty analyses were taken per specimen. The peak-to background ratio (P/B) method was used to measure the concentrations of calcium and phosphorus in each group of patients. Microcrystalline salt standards were used to quantify Ca and P as described in previous publications. All results were calculated as weight fraction percentage of Ca and P.

All spectra showed significant peaks for calcium and phosphorous. Quantitative analyses with the approach described in material and methods showed that the average Ca concentrations for the 200 determinations taken for each study group were 13.66% for fractured bone and 12.13% for arthrosic bone. In turn, the concentrations of P were 7.91% for fractured bone and 7.38% for arthrosic bone. The Ca/P ratio was 1.69 in bone corresponding to patients with arthrosis and 1.82 in patients with fracture. Differences were statistically significant for Ca, P and Ca/P ratio.

These results reveal that cancellous bone obtained from patients with arthrosis was stoichiometrically similar to the normal bone, which is characterized by a Ca/P molar ratio corresponding to hydroxyapatite (1.67). Therefore, this bone can be considered as normal from the microanalytical standpoint. However, fractured cancellous bone had an increased Ca/P ratio associated to an alteration of the Ca and P concentrations.

Cancellous bone obtained from arthrosic patients can be considered as normal from the microanalytical point of view.

This work was supported by grant SAS PI-0808-2010 from Consejería de Salud y Bienestar Social, Junta de Andalucía, Spain.

## **F.12 A FLUORESCENT STAIN OF THE INTRACELLULAR MEMBRANE SYSTEM WITH FLUOROPAQUE®**

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Area of Histology, Faculty of Medicine, and INCYL, University of Valladolid, Spain.

Fluoropaque® is a mixture of fluorescent dyes whose main utility is to stain in a general manner cells in culture on transparent or opaque supports (Gayoso, 2012). One of the fluorescent dyes is DAPI, that stains nuclei in blue, and the other one is eosin, that stains cytoplasms.

Eosin under an excitation with a 490 nm wavelength light shows a very wide fluorescence emission, approximately between 500 and 800 nm, with a maximum in the green spectrum (538 nm), but with an important emission in the red zone (around 700 nm). Eosin marks the cisternae and vesicles of the intracellular membrane system, and with particular consistency, the perinuclear endoplasmic reticulum. If images are taken in the green and red spectra, after merging them, a sharper image of the intracellular membrane system may be obtained. These images may be completed by merging them with those obtained with DAPI in blue spectrum. The results of the staining with this mixture may be relatively modified by varying the pH and differentiation in alcohols during dehydration in order to better visualize some cellular organelles.

Gayoso M.J. (2012). A fast and easy method for general fluorescent staining of cultured cells on transparent or opaque supports. *Microsc. Res. Techniq.* 75: 849-851. (P 200703132).

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### F.13 PRIMARY CILIUM IN THE EYE TELOCYTES

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<sup>5</sup>Department of Surgery, Gynecology and Obstetrics. Section Stomatology. Faculty of Odontology. University of Huesca, Huesca, Spain.

Eye is a highly organized and complex structure. The fine structure of eye has been previously studied using the electron microscope (EM) but EM has recently made possible the identification of a new interstitial cell: the telocyte (TC) in sclera, limbus and uvea in the mouse eye. TCs are found to be in close relationship with nerve endings, blood vessels and different types of resident cells, suggesting a role of telocytes in complex intercellular signaling.

We have identified a relevant feature in these cells, the presence of a single non-motile cilium called primary cilium. Primary cilia contain a 9+0 axoneme, consisting of nine outer doublet microtubules but lacking the central pair of microtubules that is found in the 9 +2 axonemes of most motile cilia. Primary cilia are sensory cellular antennae that coordinate a large number of cellular signaling pathways (Satir and Christensen, 2008), which could confirm its important role in cell signaling.

Eyes from four C57BL/6 mice (12 months old) were used for the ultrastructural study. Transmission electron microscopy (TEM) was performed on 60 nm thin sections using a Morgagni 268 electron microscope.

In all specimens studied we have observed the presence of telocytes (TCs). TCs have a small cell body and very long and thin cell prolongations called telopodes with moniliform appearance, dichotomous branching and 3D-network distribution. A primary cilium is observed on the surface of the telocyte and exhibits a 9+0 pattern.

The primary cilium of telocytes associated with the different locations in the eye may be particularly important in signaling processes within the different cellular niches.

## **F.14 MORPHOMETRIC COMPARATIVE STUDY OF DERMAL COLLAGEN BUNDLES DURING SKIN DEVELOPMENT, MATURATION AND AGING**

Marcos-Garcés V<sup>(1)</sup>, Molina Aguilar P<sup>(1)</sup>, Bea Serrano C<sup>(1)</sup>, García Bustos V<sup>(1)</sup>, Ferrández Izquierdo A<sup>(1,2)</sup>, Ruiz-Saurí A<sup>(1,2,3)</sup>

<sup>1</sup>Department of Pathology, Faculty of Medicine and Odontology, University of Valencia

<sup>2</sup>Hospital Clinic Research Foundation Valencia-INCLIVA

<sup>3</sup>Biomedical Research Networking center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN)

The collagen fibers constitute the main component of the dermis and play an important structural and functional role at the tissular level. The aim of this work is to objectively quantify the changes in the organization and structure of these fibers that take place during the lifetime, in order to establish their role in the processes of skin development, maturation and aging.

45 autopsic skin samples of different ages were obtained, from 2 months and 95 years (mean age = 57.01 years); we used a blind strategy to mask the age. Samples were stained with Masson's trichrome. Five pictures were taken with a 400x magnification of the papillary dermis and with a 200x magnification of the reticular dermis, for each case. In each picture, the area filled with collagen, the collagen bundle thickness, the thickness of the papillary and reticular dermis and the orientation of collagen bundles in the reticular dermis by Fourier analysis were measured. Image-Pro Plus 7.0 software was used. The mean of all five pictures was used to calculate the mean of each case. Statistical analysis was performed with SPSS 17.0 software, and included lineal, quadratic and cubic regressions. A p value < 0.05 was considered statistically significant.

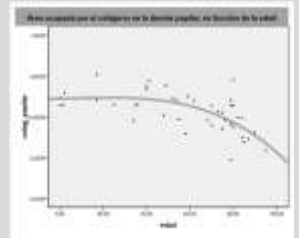
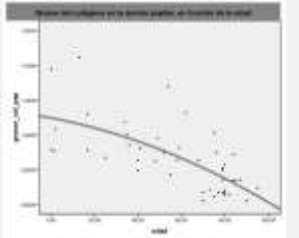
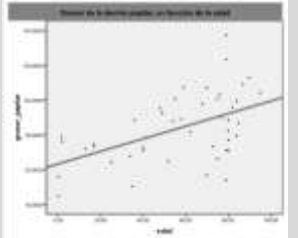
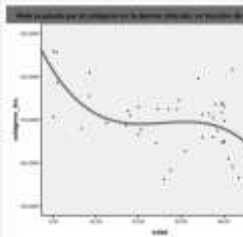
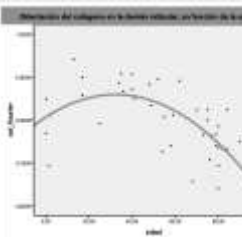
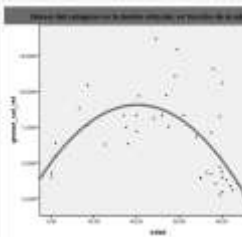
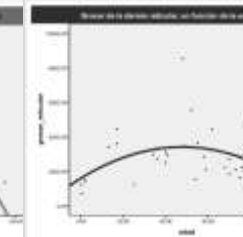
In the papillary dermis, the area filled with collagen significantly decreased with age (p<0.0001), and also the collagen bundle thickness (p<0.0001), fitting a cubic and quadratic regression models, respectively. The thickness of the papillary dermis significantly increased with age, fitting a lineal regression (p<0.0001). In the reticular dermis, cubic regression showed a significant decrease in the area filled with collagen as a function of age (p=0,002); in this location, both the collagen bundle thickness and the orientation of this fibers fit a quadratic regression model as a function of age, with statistically significant results (both p<0.0001). The thickness of the reticular dermis also fit a quadratic regression model as a function of age (p=0,011).

Our study proves a loss of thickness in dermal collagen bundles (suggestive of fiber degeneration) and in the area filled with these fibers in the dermis (suggestive of loss of density) during the lifetime. In the reticular dermis, collagen bundles arranged in a parallel fashion in the first and last life stages, suggestive of tissue immaturity and degradation, respectively - but in a three-dimensional and disorganized fashion in the adulthood. The thickness of the reticular dermis decreases with age, but increases with age in the case of the papillary dermis. These results constitute a baseline data to further investigations and contribute to explain the morphologic changes in skin that are associated with age.

Moragas A, García-Bonafé M, Sans M, Torán N, Huguet P, Martín-Plata C. Image analysis of dermal collagen changes during skin aging. *Anal. Quant. Cytol. Histol.* 1998 Dec;20(6):493–9.

Kohl E, Steinbauer J, Szeimies R-M. Skin ageing. *Journal of the European Academy of Dermatology and Venereology.* 2011 Aug;25(8):873–84.

Naylor EC, Watson REB, Sherratt MJ. Molecular aspects of skin ageing. *Maturitas.* 2011 Jul;69(3):249–56.

<b>Table 1: statistic results and graphics of the morphometric parameters and its variation as a function of age</b>				
<b>PAPILLARY DERMIS</b>				
<b>Parameter (as a function of age)</b>	<b><i>Area filled with collagen</i></b>	<b><i>Collagen bundle thickness</i></b>	<b><i>Thickness of the papillary dermis</i></b>	
<b>Regression model</b>	Cubic	Quadratic	Lineal	
<b>R<sup>2</sup></b>	0,437	0,461	0,26	
<b>p value</b>	<0,0001	<0,0001	<0,0001	
<b>Scatter plot</b>				
<b>RETICULAR DERMIS</b>				
<b>Parameter (as a function of age)</b>	<b><i>Area filled with collagen</i></b>	<b><i>Orientation of collagen bundles</i></b>	<b><i>Collagen bundle thickness</i></b>	<b><i>Thickness of the papillary dermis</i></b>
<b>Regression model</b>	Cubic	Quadratic	Quadratic	Quadratic
<b>R<sup>2</sup></b>	0,303	0,408	0,433	0,155
<b>p value</b>	0,002	<0,0001	<0,0001	0,029
<b>Scatter plot</b>				

### **F.15 CO-LOCALIZATION OF CELLULAR ANTIGENS AND EXTRACELLULAR MATRIX COMPONENTS USING DIGITAL IMAGE ANALYSIS OF SERIAL SECTIONS. A MODEL IN NEUROBLASTOMA**

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Innovative imaging technologies are revolutionizing the life sciences. Recent developments enable scientists to visualize and thereby better understand the process that govern life and disease in cells and organisms. Digital image analysis can be applied to the tissue elements involved in tumor microenvironment to better describe this field.

To develop tools that let us quantify the different cellular and non-cellular stroma elements and the relationship between them, using a neuroblastic tumor model. This is, therefore, a functional and active perspective.

Digital microscopy was used in the field of quantitative histochemistry and immunohistochemistry of biomarkers related to the immune system cells (CD8, CD20, CD45, CD11b, CD11c, CD68 and CD163), vascular structures (CD31, D2-40, CD105) and extracellular matrix (Gomori, Masson's trichrome and alcian blue) in ten neuroblastic tumors. Image analysis was performed using different softwares to properly segment the different markers. The binary images or masks generated from the image analysis, which represent the quantified marker, can be overlapped, thus enabling the co-localization of the markers.

We describe the overlapping zones as pattern classifiers of immune cellular organizations, perivascular niches and extracellular matrix scaffolding systems.

Interrelations between cells and extracellular matrix structures can be defined using imaging technologies. The achievements and measurements in the digital pathology field increased the modelling of patient-tumor-microenvironment interactions.

Grants: ISCIII2012-RED-36-020, ISCIII2010-PI00015.

## G. Teaching

### G.1 ¿PUEDE SER ATRACTIVA UNA CLASE DE HISTOLOGÍA?.

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Departamento de Anatomía e Histología Humanas, Universidad de Salamanca

Cuántas veces hemos oído decir a un profesional, al referirse a una asignatura, que es un “rollo” o “que asignatura más fea” y a la vez ser replicado por otro compañero diciendo: pues a mí me parecieron bonitas. Esta discrepancia se debe a que cada uno la estudió impartida por distintos profesores. Es decir: es el profesor el que hace una asignatura bonita o fea.

En esta ponencia trato de exponer cuales son las causas por las que esto sucede. Una asignatura se hace bonita cuando el profesor está motivado, conoce bien el contenido, se preocupa de actualizarlo y lo expone de modo ameno. Que esto supone mucho trabajo y que no se valora es cierto, bueno, no se valora por los organismos oficiales y nuestras autoridades, pero los alumnos lo valoran mucho, claro que eso no cuenta.

La trascendencia que esto tiene puede ser enorme, no sólo para el devenir de la disciplina en los planes de estudio sino en facetas insospechadas de la vida del futuro profesional.

Al tomar la decisión sobre donde lanzar la bomba atómica sobre Japón, el Secretario de la Guerra, Henry Stimson, eliminó Kyoto de la lista por su significación cultural (contra la opinión de otros miembros del proyecto). Se añadieron un par de objetivos nuevos, Nagasaki y Niigata, para reemplazar a Kyoto.

Henry Stimson había estudiado en la Universidad de Yale donde, un profesor español, le había fascinado con la cultura japonesa centrada en Kioto.

## G.2 USE OF AN AUDIENCE RESPONSE SYSTEM (EDUCLICK) IN MICROSCOPY PRACTICAL SESSIONS

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Information and communication Technologies (ICTs) provide tools that allow us to obtain information about student understanding during the classes. One of this tools is the audience response system (ARS) considered as promoter of active learning, something so demanded in the present organization on higher education in the EHEA.

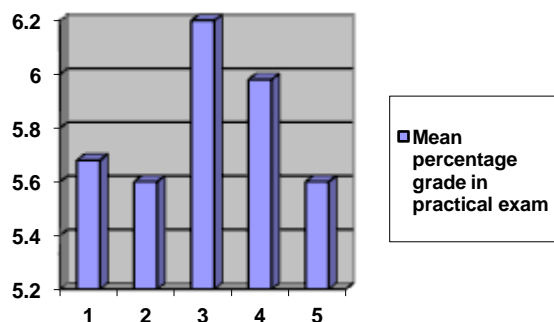
In Histology teaching, microscopy practical sessions constitute an essential and necessary activity for the understanding of the subject. During these sessions students should be able to perform the identification and description of tissue preparations.

Our organizational structure is as follows: prior to the practical sessions the students have available a script of these in the virtual campus (Moodle) with information content, material to be used and commented images of the tissue samples. The student must become acquainted of the content before the start of classroom session.

In order to know if the students reach the objectives set in each practical session, increase participation and improve retention, we used the Educlick ARS in microscopy practice groups of two subjects of first course of medical grade. We developed questionnaires in the virtual campus (Moodle) with images taken from microscopic samples that students displayed during the practical classes. There were two types of questionnaires, one of them was performed at the beginning of the session to assess prior knowledge and the other at the end to see if the students correctly identify the different microscopic structures and thus achieve the objectives of the session. Educlick system provides information on the number of students who answer questions correctly, hit rates for questions, hit rates by group, correct answers, incorrect answers, questions unanswered in each group, etc. ... all of this allows the teacher to be able to identify difficulties quickly and calibrate overall student understanding of practical session content.

A questionnaire was delivered to students near the end of the course to know their perceptions of the use of Educlick ARS in the practical sessions. The results of the assessment indicate that student opinion is quite favorable to the use of this system, majority of them are agreed that the ARS makes the class more enjoyable and participatory, and is useful for their learning.

An analysis of practical groups indicates that students of practical groups who used Educlick ARS had higher final mean percentage grade in practical exam in compare with those groups not involved in the experience.



In conclusion Educlick use offers many positive aspects as increased attention and participation of students, also professor can assess student understanding of the content of practical session and clarify points the students misunderstood.



### **G.3 INTERACTIVE LEARNING VIDEOS FOR HISTOLOGY TEACHING**

Pedrosa JA<sup>1</sup>, del Moral ML<sup>1</sup>, Hernández R<sup>1</sup>, Rus A<sup>1</sup>, Blanco S<sup>1</sup>, Peinado MA<sup>1</sup>

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We present a new multimedia teaching tool aimed to improve the reduced time that students typically spend to study the histological preparations in the laboratory. We have developed a complete description of each histological preparation used in the teaching laboratory by means of interactive learning videos, which are available to students through the Internet or a Learning Management System (LMS). The videos were performed in screencast format (digital recording from computer screen, containing an audio narration) based on images of the Virtual Microscopy section of our Interactive Histology Atlas (<http://www.ujaen.es/investiga/atlas/>). In this teaching tool, the videos are accompanied by other multimedia tools, such as self-evaluation tests and links to interactive teaching units on Histology and Organography, previously developed by the authors and available online at the website of the University of Jaén (Spain). All these components were compiled using iSpring Suite software, which generates self-learning modules, either in HTML (available online) or SCORM format (compatible with any LMS). This software can be installed as a PowerPoint option, so that all components are inserted in the PowerPoint slides, including the self-evaluation tests, also developed using an extension of iSpring Suite. Once finished the PowerPoint presentation, iSpring Suite generates a flash file, which can be directly presented to the user through a home website. It is also possible to generate a SCORM file directly with the same content and aspect. This type of file, from a LMS, provides a wide range of possibilities for interaction and monitoring by the teacher. However, both formats (HTML or SCORM) allow user an adequate feedback and send, via email, the results of the tests to the teacher and the student. The attractive interface retains all the features of the PowerPoint presentation, such as animations, transitions, videos and included web pages, as well as the interaction options in the self-evaluation tests. Moreover, it can be handled by a simple control bar.

#### G.4 “ARCHITECTURE OF TRACHEARY ELEMENTS IN TEACHING/LEARNING”

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The tracheary elements are conducting elements of the Xylem characteristic of Tracheophytes. There are two fundamental types of tracheary elements: the **tracheids** and the **vessels**. Both elements are more or less elongated cells, non living at maturity, and present lignified walls with secondary thickening and a variety of pits. The tracheids and the vessels differ from each other in that the tracheid is an imperforate cell, whereas the vessel has perforations between the different vessel members allowing their interconnection forming series of these elements and the free move of the water through these structures.

In this communication, we present a new didactic material dedicated to the study of the histology of the Xylem. This educational material contains a collection of 52 presentations with selected texts, drawings, microphotographies, animations, bibliography, and web links to different histological documents. Its content present a coherent and updated view of the study of the xylem: structure of tracheary elements (OM, TEM and SEM), the differentiation and programmed cell death, the lignifications, the phylogenetic specialization, and the structural basis of evolution in tracheary elements.

The didactic material presented here was specially created for the subject of “Cellular Biology and Animal and Plant Histology”, included in the new Degree in Biology at Complutense University of Madrid (UCM), and fully covers the new European credit included in the European Higher Education Area. In order to facilitate its use, it is presented in PDF format, and represents a support script for both the professor lectures and the particular study of each student. After being uploaded to the web of the UCM Virtual Campus, this material has been successfully used during the 2012/2013 course. The final goal of this work is update the teaching/learning process on the histology of Xylem.

Key words: xylem, tracheids, vessels, vessel members

**G.5 CATEGORY: MEDICAL PROFESSIONALISM IN THE EDUCATION OF PHYSICIANS-IN-TRAINING. A PRELIMINARY STUDY.**

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Professionalism is a basic core competency of physicians that should be taught and assessed during the residency period. Several studies suggest that achieving medical professionalism among health personnel is paramount for reaching a consistent improvement in health care. Clinical and humanistic competences are core characteristics of medical professionalism. Focusing on humanistic competences, professionalism is defined by three main components: teamwork ability, lifelong learning, and empathy. Three main curricula were described as constitutive parts of the whole medical learning process: formal curriculum, described as a planned program that includes contents, objectives and learning experiences; informal curriculum, that refers to lessons that are not explicitly taught and come mainly from social interactions during the clinical practice; and hidden curriculum, that refers to all that is taught from the organizational culture and institutional environment.

Our study was focused on medical empathy. Empathy is a cognitive attribute that involves an understanding of the inner experiences and perspectives of the patients, combined with a capability to communicate this understanding to them.

This presentation summarizes a preliminary cross-sectional study to identify factors that could be affecting the medical empathy of physicians-in-training along their professional learning process at the hospital. Information was collected from 104 questionnaires from all 190 medical residents in our Hospital. Empathic interactions were measured using the Jefferson Scale for Physicians Empathy. Differences between empathic scores with statistical significant differences were found for: geographical origin ( $p < 0.05$ ), exposure to hospital environment ( $p < 0.01$ ), professional ideal models ( $p < 0.001$ ), contact with good professional examples ( $p < 0.01$ ), and successful continued education ( $p < 0.05$ ). We conclude that acquisition of medical empathy is influenced by internal and external factors not directly related with formal curriculum. These results confirm the importance of hidden and informal curricula into acquisition of medical professionalism at teaching hospitals.

## G.6 VIRTUAL MICROSCOPE IN HISTOLOGY TEACHING

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Human Histology is a basic morphological science in health sciences studies. An important part of his teaching is microscopic observation, this is performed in microscopy laboratories where each student has an optical microscope for observation and identification of different histological samples for a limited time.

Advances in computer technology have made possible the development of virtual microscope (VM) that could be defined as a computer system that allows the observation of microscopic samples on a computer screen in a manner analogous to a real microscope allowing observe, browse and annotate on virtual slides. These days VM use is widespread and even in some universities is replacing the use of traditional optical microscope.

Identification and interpretation of images are basic skills in Histology that are learned and perfected with practice, for it is useful to create situations that promote this learning outside the classroom schedule. By all of this, we design an activity with the use of a virtual microscope.

The activity started in this academic year in two subjects at the Faculty of Medicine and we set it as a complement to classroom practice activity and as a practical work that students have to submit as part of their final evaluation. We used a 3D scanner Histech-Pannoramic Desk, and instrument that scans microscopic preparations in high quality. We used histological samples selected from those used during the practical sessions. The scanner creates digitized preparations that can be opened with a free program, Pannoramic Viewer, that students must be downloaded to their computer.

For non-attendance practical activity, students are given a document through the virtual campus that indicates what to do on each of the digitized samples. To perform this activity the student must carefully observe the image, remember that seen in the practical sessions, consult information about the microscopic structure and later select, in the image, the desired area and make a description of it. With all of this we propose the realization of a didactic activity that can be able to develop skills that are an inherent part of the discipline such as describe, identify, compare, classify, explain, define and interpret. It also encourages independent learning outside the microscopy room allowing flexible time and space, as well as the acquisition of competences like capacity for analysis and synthesis, problem solving, decision making and self-learning, following the guidelines of the EHE.

To assess learning efficacy with use of the VM, student performance on histology practical exam was compared from one year prior to the use of VM. We observed how the grade for the histology practical exams went up from an average of 4.8 to 6.6 when compares with the previous course.

Additionally, to assess the student perceptions with the use of VM a questionnaire was performed. An overview of the responses showed that a majority of students are agreed or strongly agreed that facilitates the understanding of microscopic structures and the understanding and learning of the subject.

## **G.7 CLINICAL PROJECTION IN A PRACTICAL SESSION ABOUT HISTOLOGY METHODOLOGY**

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One of the practical competences of the new medical curriculum that interests the Medical Histology is CE20, that comprises “Learning how to handle laboratory equipment and perform basic techniques”. To fulfill it, a practical session was designed taking into account two premises: 1. the histology knowledge should have a clear clinical projection and 2 students should play an active role.

The present study was designed to determine if first year students become aware after the practice of the clinical projection that histological methodology has. Therefore, it lets us analyze the efficacy of the practical session design.

A test with 10 items was elaborated and two topics were evaluated: ability of the students to select the proper methodology in a given clinical situation (1-5) and knowledge of the principles of laboratory techniques (6-10). Both topics were analyzed independently among Medical Histology I students who have attend the practice (n=63) vs those who have not attend the practice yet (n=62). The same test was provided to first year resident physicians (n=39) from Hospital Universitario Reina Sofía.

Statistical analysis showed significant differences among the three groups for both sets of topics. First year students that have attended the practice get the best score in both sets, while the second place was for resident physicians (1-5 items) and for first year students who have not attended the practice (6-10 items).

a) The practical session design is efficient to translate to students the clinical application of the competence CE20. b) This practice means an improvement in medical education.

## G.8 TEACHING HISTOLOGY: ANALYSIS OF SUBJECT SPECIFIC COMPETENCES IN SPANISH PRIVATE SCHOOLS OF MEDICINE.

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Spanish universities are developing a curricular reform according with the Bologna Process. Previously, I showed that histology curricula in most public universities include a lot of subject specific competences synthesized as follows [1]:

To have a working knowledge of tissues and organs.

Diagnostic ability to identify tissues, organs and tissue components.

Ability to carry out histological techniques.

Use of scientific terminology.

Use of the microscope.

My objective was to analyze the subject specific competences of histology curricula in newly created private schools of medicine.

I have analyzed the histology curricula of the five private schools of medicine listed in Table 1 from the academic guide available at their respective web sites.

As in my previous study [1], histology specific competences were found as items under different epigraphs: specific competences, general competences, objectives, results of learning, etc.

Most of the competences could be arranged in the same five categories of basic competences previously shown in public universities. Results are shown in the table 1.

Table 1. Histology specific competences by university and comparing with previous data about public universities.

	Subject specific competence				
	1	2	3	4	5
University	Knowledge	Diagnosis	Techniques	Scientific terms	Use of the microscope
Francisco de Vitoria	X	X	X	-	-
University of Navarra	X	X	X	X	-
San Vicente Mártir	X	X	X	-	X
Alfonso X el Sabio	X	X	X	-	-
European University of Madrid	X	X	X	X	-
TOTAL PRIVATE SCHOOLS OF MEDICINE	5 (100%)	5(100%)	5(100%)	2(40%)	1(20%)
TOTAL PUBLIC SCHOOLS OF MEDICINE [1]	19(100%)	19(100%)	16(84.2%)	5(26.3%)	3(15.8%)

Competences 1 to 5 are the listed in the introduction.

Some competences, which could not be included in the previous five categories, were found in some university, e.g. introduction to the pathological knowledge (one school) and anatomical knowledge (one school).

Other competences were in fact transversal or general competences, but not subject specific competences (e.g. oral expression, writing, introduction to scientific research...).

In conclusion, Spanish private schools of medicine show the same basic histology specific competences than public universities,

The data obtained from academic guides of private schools of medicine supports my previous work about histology specific competences in public universities, where I have proposed the following three main or basic histology specific competences [1]:

Knowledge of the microscopic structure of tissues and organs, connecting with function, the basis of pathology and other biomedical subjects; analyzing and answering questions or problems using the scientific terminology.

Acquisition of diagnosis ability to identify tissues, organs, tissue structures, and basic stains, describing and drawing the sample observed.

Knowledge and performance the basic procedures to obtain histological samples.

[1] Sáez FJ (2011). La enseñanza de la Histología por competencias en las Facultades de Medicina españolas. In (Bermúdez M P and Guillón-Riquelme A comps.): *VIII Foro de Evaluación de la Calidad de la Investigación y de la Educación Superior: Libro de capítulos*. Asociación Española de la Psicología Conductual (AEPC), Granada, Spain.

## **G.9 THE USE OF THE VIRTUAL MICROSCOPE AS A TOOL FOR THE HISTOLOGY TEACHING IIN MEDICINE. A TWO YEAR EXPERIENCE**

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The teaching of histology is partially based on the microscopic recognition of the organs and tissues. Traditionally, in our practices classroom the student drew in a notebook what they viewed at the microscope. The student frequently fails to recognize its usefulness; and so, it became tedious and boring for him. The virtual microscope can help to get a more active work by the student.

Our University purchased two scanners Leica SCN400F, to scan microscope slides that were uploaded to a server computer. The students logged in to the system by a web page. They moved the virtual slide top to down and left to right, and increased and decreased the magnification easily; they also took pictures that saved in their computers. Previous to the practice session, the student downloaded a document (screenplay) from the “aulavirtual” of our University, which contains the essential information to realize the practice autonomously. During the practice session the student used a real microscope with real slides. They also could use atlas books, as well they could access to internet from their computers. The screenplay contained the objectives for each microscope slide, as well as some question to help to the student to view the details of each slide. During the practice session the teacher did not explain the slides, he only resolved some questions. Finally, at the end of the practice session some students explained to the other the different contents of the different slides, and they got a score. Later, at home, they accessed to the virtual microscope, took pictures and include them in the screenplay, adequately marked with arrows and letters. Some of these screenplays were asked to the student during the next practice session and were scored. At the end of the academic year all the screenplays are asked to all the students and scored.

In previous years, the teacher explained at the beginning of the practice session the objectives for each slides with the help of a powerpoint presentation. Most of the students had not read previously the corresponding theory explained at the classroom. Now, the teacher explain nothing, and at the end of the practice some students have to explain the slides with the help of a powerpoint presentation that was previously prepared by the teacher, or accessing to the virtual microscope. So, now, the student has to study the slides in the real microscope with his/her personal background. Now the student has previously read their theory notes, or read them during the practice session, read atlas books, talk between them, access to internet. In summary, the students do the necessary to understand the slide that they examined using their real microscope. They actively work during the practice session. Some of their final explanation about the slides are really good because they have worked on them personally. At the end of the year, they have a briefcase with all their screenplays, that is really a personal atlas book.

Does this increased their knowledge in histology? We think that the answer is yes. However, we have not quantified this, because at the same time that we implanted the method, we did more difficult exams. So the exams and the result obtained in previous years are not comparable with the present. As an example, we could inform that last year in our subject of “Cell Biology and Histology” failed 12 students, while in the present years 4 students failed, and with a more difficult exam.



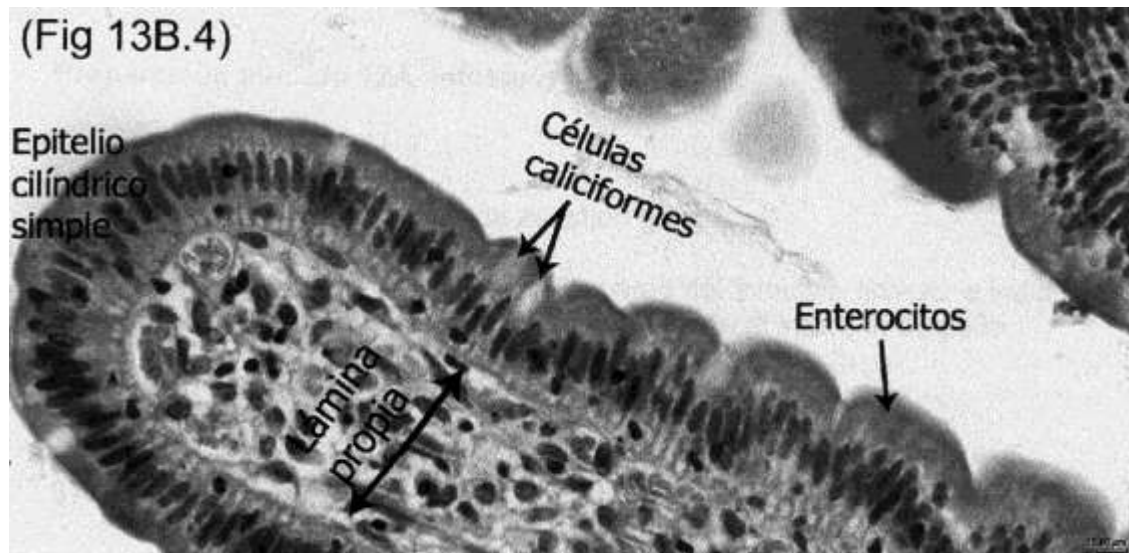


Figure 1. Example of a picture taken and labeled by a student (Ana Muñoz Tornero; with permission)

## **G.10 THE USE OF MICROTEACHING AS A SELF-REGULATED LEARNING PROCESS IN HISTOLOGY**

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Microteaching involves a simulated teaching session known as microlesson of five to ten minutes of duration in which students teach a short lecture to their classmates. Microteaching-microlearning exercises are effective methods to enhance and develop communication and critical-thinking skills in students. A microlesson is elaborated and taught by the students, who present the information to other students without systematic external guidance. For that reason, a microlesson can be categorized as educational tool in the context of self-learning. The goal of this study was to assess the perceptions of how the students who provide instruction using microteaching would design such activity and to compare these with the perceptions of the same students when they receive such instruction.

In this work, two different questionnaires were used to evaluate the perceptions of the second-year undergraduate medical students enrolled in the histology course. The question inquired in the first questionnaire was about what the students would use to prepare a microlesson. The second questionnaire investigated about what the students would use to expose a microlesson. Each questionnaire had 10 items. The responses in both questionnaires were recorded with a symmetric agree-disagree Likert-like scale on which students indicated their level of agreement or disagreement for each item. To evaluate the results, the Mann-Whitney non-parametric statistical test was used.

The lowest score corresponded to the item related to the use of the same tone of voice in the exposition ( $1.85 \pm 1.08$ ). The highest score corresponded to the item regarding the use of specific objectives to prepare a microlesson ( $4.80 \pm 0.44$ ). For preparing the microlesson, significant differences were found for the use of textbooks, internet information and specific objectives, which were higher before receiving the microlesson. In contrast, the use of a programmed index was significantly higher after receiving the microlesson. No statistical differences were found for the rest of the options analyzed.

Students consider that they would more significantly use specific objectives, textbooks and Internet information before preparing a microlesson than after its reception. This could be linked to the information that the students consider that should be incorporated to a microlesson and to the objectives that they believe they should reach before the microteaching session. Regarding the exposition, the students showed significant differences in the use of a previously programmed index and in the use of PowerPoint software.

The new teaching guidelines emphasize the need for students to engage in self-regulation of learning and practice. As the self-assessment is linked to how individuals seek and interpret feedback we consider that the use of microteaching techniques could be useful not only for self-learning but also as a tool to promote self-regulation across the curriculum.

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### **G.11 A STUDY OF THE STUDENTS' INTRINSIC, CAREER AND GRADE MOTIVATION IN PHARMACEUTICAL HISTOLOGY**

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The students' motivation to learn basic sciences in pharmacy curricula is poorly understood. The purpose of this study was to investigate the influence of different components of motivation (intrinsic motivation, and extrinsic -career and grade- motivation) on learning human histology in pharmacy curricula and their relationship with the final performance of the students in histology.

Glynn Science Motivation Questionnaire II was used to compare students' motivation components to learn histology in first-year male and female undergraduate students enrolled in the pharmacy degree program at the University of Granada.

To compare the results between male and female students we used the Mann-Whitney test. To determine if the final performance of the students was correlated with the components of motivation, we used the Kendall tau correlation test. All statistical analyses were two-tailed and values of p less than 0.05 were considered statistically significant.

For the intrinsic motivation component, the overall mean score was  $3.42 \pm 1.07$  in pharmacy students. For the career motivation component, the results were  $3.39 \pm 1.09$ . For the grade motivation component, the results were  $3.68 \pm 1.17$ . Significant differences ( $p < 0.05$ ) between genders were found for the components Intrinsic Motivation and grade motivation.

When the overall mean scores for the different components were correlated with the final performance in histology of the students, we found a significant positive correlation ( $p < 0.05$ ) for career motivation.

Investigation of the motivational profiles for learning histology in pharmacy is potentially useful to foster the students' learning process, because if they are metacognitively aware of their motivation they will be better equipped to self-regulate their science-learning behavior in histology. This information could be useful for instructors and education policy makers to enhance curricula not only on the cognitive component of learning but also to integrate students' levels and types of motivation into the processes of planning, delivery and evaluation of medical education.

Supported by Unidad de Innovación Docente of the Department of Histology, University of Granada, Spain.

## G.12 THE TYPE OF EXAM AS INDICATOR OF THE STUDENT'S LEARNING STYLES IN HISTOLOGY

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Exams can be used not only for normative evaluation, but also as diagnostic tools to evaluate how knowledge is acquired by the students. The specific capabilities and abilities fulfilled by the students can be evaluated by using different types of exam. In histology, multiple-answer examination allows evaluating the knowledge acquired by the students (quantity and quality of data and information that student can accurately handle). Other examination types such as schematic drawings that the students must carry out could be useful to evaluate the spatial coordination and display of a histological concept. Identification of key structures in pre-made schemes and drawings and microphotographs could be useful to evaluate the capability of the students to correlate histological concepts with the images (a schematic representation of a concept or a real picture of a concept). In the present work, we analyzed the response of the students to each type of exams to evaluate their recognition and recall memory.

60 students enrolled in a General Histology course at the Health Sciences Faculty of the University of Granada were included in this study. At the end of the course, a final exam was used to evaluate the knowledge acquired by the students during the whole semester. This exam had four independent parts: a multiple-answer test exam, a scheme or drawing for the students to recognize and label, a drawing that the students had to do themselves and a microphotograph that the students had to recognize and label. Each part was scored from 0 to 10. To compare the average results obtained between different parts, the Student t test was used.

The highest scores were found for the microphotograph (average 8.16) and the schematic drawing to recognize (7.95), whereas the lowest results corresponded to the multiple-answer test (6.58) and the drawing done by the students (6.85). Statistical differences were found for the comparison of the results of the multiple-answer test with the schematic drawing to recognize ( $p=0.0001$ ) and with the microphotograph to recognize ( $p=0.0000$ ) and for the comparison of the drawing that the students had to do with the schematic drawing to recognize ( $p=0.0253$ ) and with the microphotograph to recognize ( $p=0.0066$ ).

Our results suggest that students with the same background show clear differences in their academic performance to different types of exams. They obtained the highest scores when they have to identify either a schematic representation of a concept or a real picture of a concept shown as an image in the exam. Regardless the information that the students had, this could mean that students preferentially have a visual recognition memory for the histological concepts. This recognition memory capability is significantly higher than their recall memory capability as determined by the multiple-answer exam type and the schematic drawings that the students had to carry out. These results imply that images should be incorporated to the exam in histology in order to reinforce recognition significant knowledge.

Supported by Unidad de Innovación Docente of the Department of Histology, University of Granada, Spain.

### **G.13 SCIENTIFIC FOREIGN LANGUAGE SKILLS OF SPANISH MEDICAL STUDENTS. A COMPARATIVE ANALYSIS OF ENGLISH, FRENCH AND SPANISH**

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The knowledge of foreign languages (FL) is an important competence in medical and scientific careers, because most of the scientific information is in English or French in Europe. In addition, acquisition of scientific terms and knowledge in a FL is difficult and it is not widely considered in the basic and university curricula of medical students in Spain. In this sense, in this work we performed a histology test in English, French and Spanish in Spanish medical students in order to determine the knowledge of these terms in FL.

This study was performed with voluntary medical students enrolled in their first year at the Faculty of Medicine of the University of Granada. We developed a histology test in English (n=231), French (n=202) and Spanish (n=252). Each test had ten multiple choice questions related to the course of practical histology. The English and French tests were performed using the application of [www.thesistools.com](http://www.thesistools.com). In the case of Spanish, the test was the final practical exam of the course.

For each test, we calculated the scores reached by each student from 0 to 10. To compare the results between the three languages and between male and female students, we used the Mann-Whitney non-parametric test. All statistical analyses were two-tailed and values of p less than 0.05 were considered statistically significant.

The mean score of the histology tests were  $6.32 \pm 1.93$  in English,  $4.45 \pm 2.32$  in French and  $6.63 \pm 1.5$  in Spanish. We observed significant differences between English v/s French and Spanish v/s French ( $p < 0.05$ ). However, no significant differences were found between English v/s Spanish ( $p = 0.114$ ).

In relation with gender, we found the same results: male and female students showed significant differences between English v/s French and Spanish v/s French ( $p < 0.05$ ). Nevertheless, no significant differences were found between English v/s Spanish for male ( $p = 0.49$ ) and female ( $p = 0.14$ ). Analyzing each language separately, we only found significant differences in the Spanish test between female v/s male ( $p < 0.05$ ).

This type of study allowed us to confirm that our medical students have a better understanding of English as a FL in comparison to French. In addition, the lower results obtained by our students in the French test could be related to their poorer knowledge of this FL. The analysis by gender demonstrated that male and female students have a similar background of each FL, English and French. However, the differences between genders were evident in their mother tongue, Spanish. Finally, the progressive application of this type of tests could be a useful tool for the passive acquisition of scientific terms in FL. In conclusion, these results suggest that specific medical French courses should be offered to the medical students in order to improve their capability to understand scientific papers in French.

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## **G.14 REFLECTIVE PRACTICE IN PROGRESS II. OUR EXPERIENCE AT UNIVERSITY LEVEL: ANALYSIS OF RESULTS**

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The aim of this paper is to present and analyse the results of the application of “Guided Weekly Reflection Papers” (GWRP) in our teaching practice during the two last academic years. This strategy has been implemented by our group of Teaching Innovation, composed by teachers of different areas of the University of Alcalá along five years in which we have introduced several motivating modifications based in the experience we have acquired. The research described here is oriented to analyse two main different aspects: on one hand the percentage of the students’ participation in this activity in the academic course 2012-13 as much as where in the study plan is located the considered subject. The second aspect to analyse would be the level of satisfaction of the participant students as much as the usefulness of this tool and how has it influenced the performance in their learning process. The outcomes of data analysis show that students evaluated positively the experience and prove that GWRP tool provides a higher engagement of the students with the teaching-learning-process allowing helpful learning environment

**Key Words:** Guided weekly reflection papers (GWRP), reflective learning process, university teaching innovation

**G.15 MICROSCOPY: TRADITIONAL OR VIRTUAL?  
WHAT STUDENTS SAY?**

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The visual aids for learning clearly advanced over the last several decades. With the approach digital slice imaging there is an opportunity to complement the way of teaching and learning histology. The use of this technology was used into Oral Biology course at CEU-San Pablo University. The aims of this study was determinate the student opinion with the use of traditional versus virtual microscopy.

In order to know what students say about traditional or virtual microscopy, we carried out a survey at 88 students of Oral Biology, who during the previous year had studied Biology with us. There were 12 items with 5 possible answers each.

Students pointed out that virtual microscopy have ability to standardize image, allows to work outside the laboratory, facilitates the active involvement in learning, allows understanding concepts and increasing the interest in the subject.

Virtual microscopy is a good tool that provides mobility, portability (time and location), and convenience to students. However, we believe that the students should know traditional microscopy.

## **G.16 HISTOLOGY IN NURSING TRANSDISCIPLINARY TEACHING AND LEARNING: VIDEO AS A TOOL FOR CONTENT INTEGRATION**

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ASFE, The Teaching Innovation Group for Nursing, was born to promote the acquisition of generic and specific competences on nursing students and to stimulate curriculum integration among the basic disciplines, including Cytology and Histology, and the clinical ones. The achievement of this goal requires the design of innovative strategies and new educational materials, involving the most updated technical, scientific and methodological tools. In this way, digital media help to provide a context for the teaching-learning process to develop participatory dynamics, to encourage autonomous learning, to reinforce the role of students in their own learning process and to promote analytical thinking and knowledge questioning focused on the training of critical professionals. Nevertheless, to reach didactic excellence the production of audiovisual tools must be preceded by a careful planning and an accurate design.

The UAH Innovation Group on Nursing choose "*Memory*" as the subject for making an interdisciplinary documentary that should be published along with a booklet, containing the activities that must be implemented in the classroom. The production schedule to bring it to completion followed no less than nine different stages:

Researching: Select the appropriate contents and strategies to communicate the subject in terms of audiovisual storytelling, keeping the adequate balance between a highly accurate scientific content and a clearly understandable pedagogical approach.

Writing: Shaping contents into an audiovisual format, developing an audiovisual script.

Preproduction: Production planning according to the conceived script. Planning the shooting, build the shooting team, select the interviewees on Universities and Health Institutions, pick up the locations, design animation, work on the production and direction breakdown, establish the production plan.

Review: Correcting and refining those aspects that could be improved with the help of scientific and technical experts.

Shooting: Recording no less than forty hours of HD raw material with two different cameras all along the spanish geography, including air and underwater shots.

Sound Design: Locution and soundtrack recording.

Editing: Real life, animation material, written mentions and soundtrack compiled in a one hour documentary.

Publishing: DVD copies, covers and booklet made for massive distribution.

After the first screenings the students view offered a very positive impression of the video as a learning tool (table I). It is worth to mention how the audiovisual approach helped to increase their motivation and interest in the discussed subject, to unveil new concerns and to ease the interdisciplinary approach. However, if we expect students to grant the documentary a status similar to a textbook an additional effort must be made by teachers in order to develop the close linking between the audiovisual discourse and the syllabus content of the different matters involved in the experience.

From our experience, this kind of audiovisual tool, carefully researched, written, planned, produced, recorded and edited overcomes the many limitations of the customary textbooks in



Histology as well as in other disciplines. On the other hand, Histology, understood in the context of transdisciplinarity, acquires a new entity. In traditional University education, the different matters of the curriculum are often considered as isolated and unrelated subjects, thus promoting a kind of learning lacking meaning and context. On the contrary, contents integration makes learning more solid, helping knowledge retention to last more, to be recovered more easily and to be brought out in new situations and different contexts as a *supraorganized* learning which it will undoubtedly be useful in their future careers where continuing training throughout all their lives will be the primal requirement to keep and enlarge their professional skills.

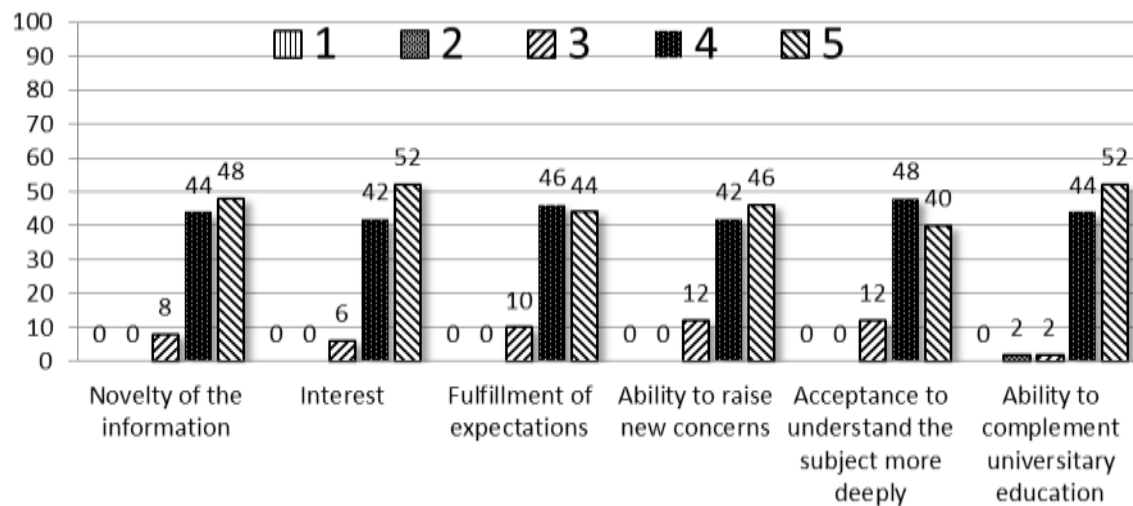


Table I: Opinion of the students after the screening of the video (valuation according to a Likert scale of 1 to 5 (1. Strongly disagree, 2. Disagree, 3. Neither agree nor disagree, 4. Agree, 5. Strongly agree)).

## G.17 HISTOLOGY IN CONTENT INTEGRATION IN NURSING

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*Integrated Tutoring*, developed during three academic courses in UAH Degree in Nursing, is based on the principle of educational intervention to help students to “*learn how to learn*”, in order to develop their general and specific skills that enable them an autonomous learning in the future. The main objective is to promote contents integration among the different disciplines included in the first year curriculum of the Nursing Degree, being Histology one of the basic disciplines. This new way of teaching and learning will allow the student to reach significant transversal competences (synthesis, presentation, information search, workgroup, etc.). The student, throughout their work in cooperative groups, becomes the protagonist of his own knowledge building.

Ensure significant learning and content integration in a time of accelerated social and technological changes requires the continuous and extended teaching effort for innovation in redesigning professional profiles and methodological strategies. In the Nursing Degree, basic disciplines provide the cognitive structure and the foundations in which the future nurses can anchor its response and intervention skills so it is essential for them the integration of the different matters to be able to face new situations in different conditions.

However, through *Integrated Tutoring*, an integrative and transdisciplinary learning tool, barriers among histology and the remaining disciplines of the curriculum are demolished, so students learn in the same way as they will confront in the future their careers, contextualizing and integrating knowledge. In this way, during the two semesters, students groups work on situations designed to be approached from the different disciplines of the curriculum, interweaving their contents and overcoming compartmentalization.

At the end of each academic year, the students opinion is surveyed in order to measure their satisfaction with the activity, evaluate if this activity allows the acquisition of the generic competences of the Degree (table I), and find suggestions in order to improve the activity in successive experiences. All items were highly valued, but the integration of different matters, the analysis and synthesis capabilities, the improving skills for generating new ideas and the teamwork itself were specially praised.

On the other hand, this activity was also valued in a very positive way by the teachers of the different matters underlining the students increasing ability for content integration and the substantial progress experienced in communication skills. This involvement will allow the students to cope with the demands of continuous training throughout their careers, enabling them to become adroit professionals in a world in constant change.

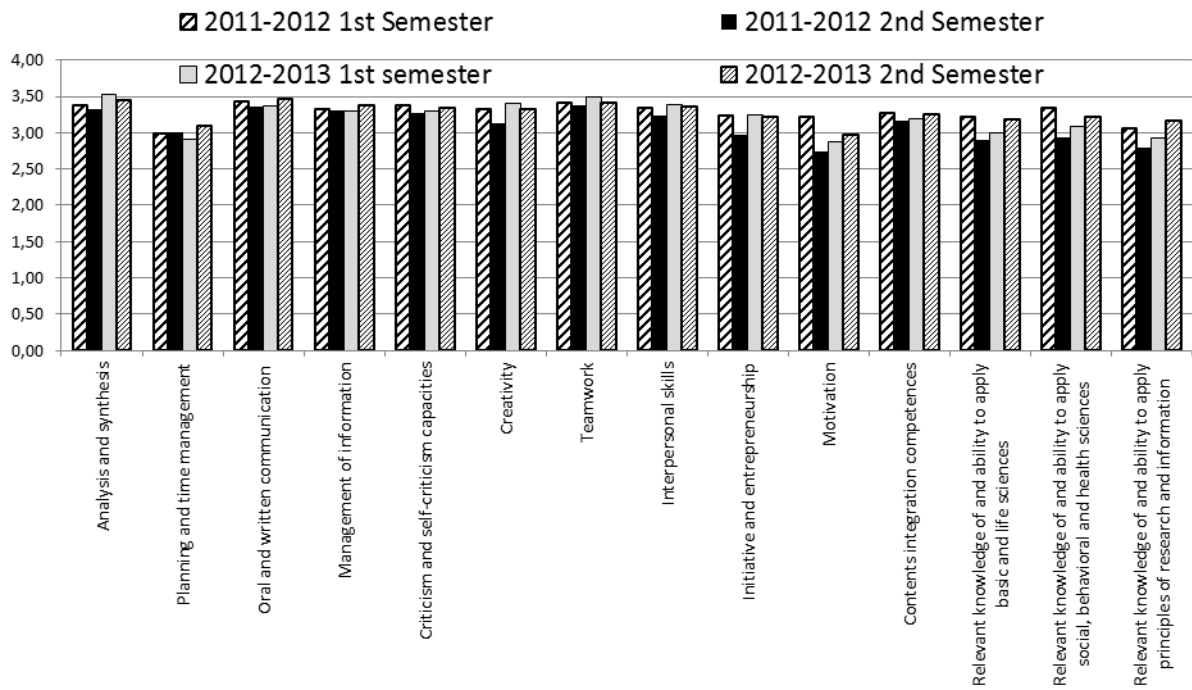


Table I: Students assessment on the competence degree achieved through *Integrated Tutoring* according to a Likert scale of 1 to 5 (1. Strongly disagree, 2. Disagree, 3. Neither agree nor disagree, 4. Agree, 5. Strongly agree).

## H. Comparative Histology

### H.1 GLIAL CELLS IN NATURAL PRION DISEASES

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Transmissible Spongiform Encephalopathies (TSEs) are a group of neurodegenerative disorders for which no effective treatment is available to date. They are mainly characterized by vacuolation and accumulation of pathological prion protein (PrP<sup>sc</sup>). Gliosis has been widely described as another lesion always present in them, but few efforts have been made to go into the role of these cells in TSEs in greater depth.

The overall objective of this work is to assess the actual role of glial cells as components of the protective system in the TSE pathogenesis. With this aim, histological and immunohistochemical techniques have been carried out on sagittal sections corresponding to cerebella from affected and non affected cases.

Specific changes associated with distribution and morphology of glial cells in relation with neurons are described in the present study. The involvement of these host cellular populations has been therefore evidenced here.

All advances attained in the frame of this approach result especially relevant, not only for TSEs study, but also for other neurodegenerative diseases such as Alzheimer or Parkinson, whose molecular basis and mechanisms of propagation have been recently demonstrated to be shared. Whether glial population has a role in the progress of the neurodegenerative progress, it could constitute a possible target for alternative therapeutical strategies not considered to date.

Acknowledgments.- This work was funded by a grant from the University of Zaragoza (UZ2010-BIO-10).

## H.2 COMPARATIVE STUDY OF BLOOD TISSUE FROM INVERTEBRATES TO VERTEBRATES

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Blood tissue possess an important function because it presents a characteristics of been fluid that allows to communicate the whole body. This property is unique among the others tissues on the multicellular organisms, in vertebrate it calls blood and in invertebrates calls hemolymph. This tissue is composed by two major parts, the liquid phase is formed by proteins, nutrients, waste hormones among others and the cellular phase presents cells which in turn are divided in three groups: erythrocytes has the function of transport oxygen, thrombocytes participate in the coagulation system and leukocytes constituents immune mechanism which is relevant for the survival of the organism. Mammals erythrocytes are enucleated cells about 7  $\mu\text{m}$  of diameter while in the rest of the other vertebrates shows a bigger size, has elliptical shape and present nuclei, the red blood cells size in amphibian and fish organism are approximately of 10 x 14  $\mu\text{m}$ , in birds and reptiles are about 12 x 20  $\mu\text{m}$ . The invertebrates do not present erythrocytes because the function for transport oxygen is carried out by a specialized protein calls hemocyanin. On the other hand the platelets are only present in mammals, they are cellular fragments and participates in the coagulation system. Others vertebrates show thrombocytes, these presents the same function than in mammals but the difference is that in these animals are complete cells. However in invertebrates don't exist a specific cell that participated in the coagulation mechanism, this process is carried out by a specialized protein calls coagulin. Leukocytes participate in the defense mechanism against pathogens, these cells are present in all vertebrates and they are subdivided in five cell types, the most important difference present in these cells between vertebrate's species are the size. Mammals, amphibians and bony fish present neutrophils and the cartilaginous fish, birds and reptiles shows heterophils, both cells carried out the same function. However, heterophils possess elongated granules with acid reaction to Wright staining that gives an orange coloration. The other cell types are eosinophils, basophils, monocytes and lymphocytes; all these cells show the same characteristics in vertebrate's group. Invertebrate's defense cells are called hemocytes, these have been denominated analogous to leukocytes in vertebrates because they participate in defense mechanisms know as "Innate immune response " unlike vertebrates having innate and memory defense mechanism. Hemocytes have the ability to produces propheloxidase, this protein is produced in the interior of cell and released as phenoloxidase to hemolymph participating in the immune mechanism, and this protein is exclusive of invertebrates. Therefore, all organisms possess a blood tissue that carried out same functions by using different strategies, however these cells present different characteristics according to their adaptation environment, resulting in specific developmental characteristics of organisms.

### **H.3 EFFECT OF IL17 ON INVASION OF CHICKEN CAECAL LAMINA PROPRIA BY CRYPT EPITHELIAL CELLS INFECTED WITH *EIMERIA TENELLA***

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*Eimeria tenella* is a significant cause of disease in food-producing chickens worldwide. It is an intracellular obligate protozoan parasite having a life cycle of 7 days, during which it develops through characteristic stages confined to the caeca. The cycle involves detachment of the infected cells, which harbor the schizont stage, from the epithelium lining the lumen of the crypts of Lieberkhün to migrate into lamina propria (LP). Schizont maturation takes place as the host cell harboring the stage migrates through the LP deep to the muscularis mucosae (MM). The ability of parasitized cells to detach from the epithelium and invade the LP relays on a cascade of molecular events involving a finely tuned interplay between parasite and host cells. Among the factors playing a role, Interleukin-17A (IL17A) has been reported to activate epithelial cell contractile machinery, cytoskeleton rearrangements and tight junction disruption. These properties of IL17A have led us to study its effect on schizont maturation.

One-day old chickens were divided into 3 groups. At 15 d of age, chickens in groups I and II were infected with *E. tenella* sporulated oocysts. At 3, 4, and 5 d post infection (pi), chickens in group I were intravenously injected with anti-IL17 Ab (3 µg/bird) and chickens in group II with sterile PBS. Group III served as noninfected-noninjected control. At 5 d pi, caeca samples were removed to visualize schizonts and blood was collected to quantify IL17 in serum by ELISA. IL17 levels in infected-noninjected chickens were strongly increased compared with those in noninfected-noninjected chickens. However, in infected chickens that were given anti-IL17 Ab, IL17 levels were significantly decreased compared with those in control chickens, infected-noninjected and noninfected-noninjected.

At day 5 pi, when large numbers of schizonts are present in the caeca mucosa, schizonts within epithelial cells were identified by expression of fibronectin. In infected chickens that were not given the Ab, schizonts reached the MM, which in places was disrupted and schizonts extended into the submucosa (SM) (Fig.1A). Those migrating schizonts were mainly immature. It is to note that the LP and SM contained prominent dense infiltrates of cells of the immune system. However, in infected chickens given anti-IL17 Ab, the LP contained slightly cell infiltrates. In addition, epithelial cells harboring schizonts did not extend into the SM. The MM was observed as a continuous line and schizonts were near the epithelium lining the caeca lumen (Fig. 1B). Those schizonts were both immature and mature.

In the present investigation, we provide evidence that treatment with IL17A-neutralizing antibody led to reduced motility and reduced migration of cells harboring schizonts to deep areas in the LP, demonstrating dependence of parasitized epithelial cells on IL17 for schizont maturation.

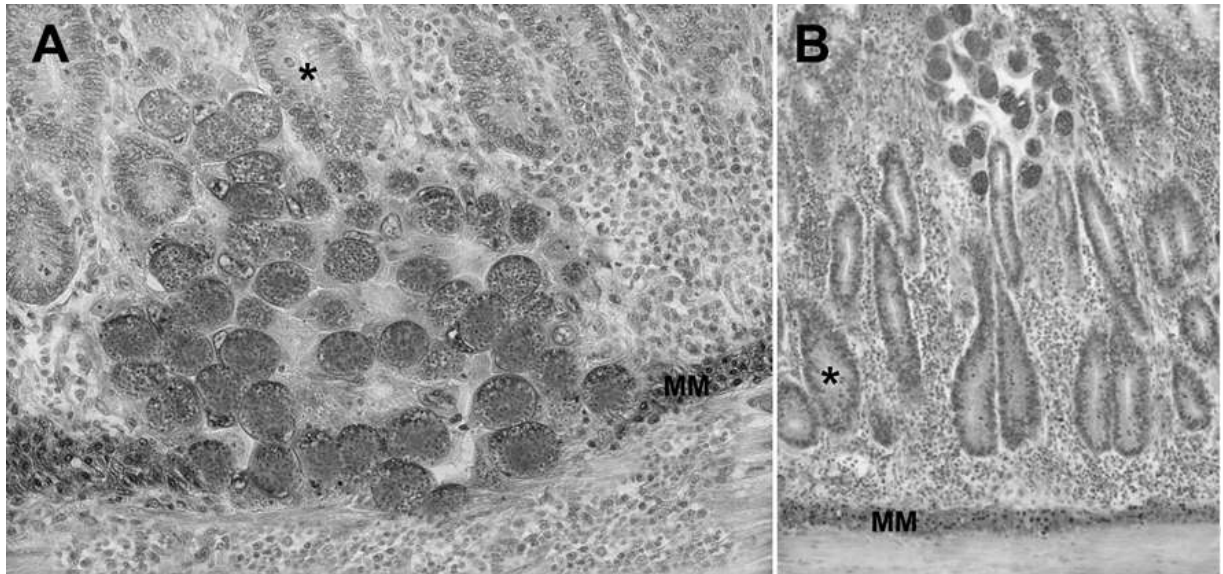


Fig.1. Sections of caeca wall showing schizonts at 5 d pi. *E. tenella* infected chickens were not given (A) or given (B) anti-IL17 Ab. Crypt lumen (\*). Muscularis mucosae (MM).

Investigation funded by Grant A46 Research Council of Aragón and USDA-CSREES 2005-01812 (US Veterinary Immune Reagent Network)

#### **H.4 HISTOMORPHOMETRIC STUDY OF GOAT ABOMASUM DURING PRENATAL DEVELOPMENT**

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Ruminant animals are born with a stomach that is structurally and functionally similar to non-ruminant animals. During the prenatal life, their stomach suffers some morphological changes to suit function in postnatal life. These morphological changes will be associated to the growth and development of gastric viscera.

The aim of this work was to study the growth of each of the tissue layers and the abomasum wall by histomorphometric analysis and mathematical models fit growth in order to obtain formulas applicable to embryonic gastric growth.

A total of 140 goat embryos and fetuses, from the first prenatal stages to birth were sampled. Specimens were divided into 5 sequential groups: group I (crown-rump length[CRL] 1.5-4.3 cm, 1-25% gestation), group II (CRL 4.4-8 cm, 25-35% gestation), group III (CRL 9-17.5 cm, 35-50% gestation), group IV (CRL 18-32 cm, 50-75% gestation), and group V (CRL 33-47 cm, 75-100% gestation). Histological sections were viewed through a microscope equipped with a digital video camera. Digital images were analyzed using the Nis-Element 2.30 software package. Variables studied were height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis and serosa) and total wall thickness. One hundred measurements were made in each tissue layer. Tissue growth models were created using four mathematical models: linear, polynomial, logarithmic and exponential. The goodness of fit of each adjustment was measured using the linear coefficient of correlation,  $r$ . In all cases, embryo body length (in centimetres) was used as the independent variable; the thickness of each tissue layer served as dependent variable.

The growth of epithelium followed a continuous involution process from early embryonic stages to birth. Its growth rate was adjusted to a polynomial type model.

The growth dynamic of the lamina propria and submucosa was fitted a polynomial type model. These tissue layers both together were increasing in thickness as the gestation period was progressing. The growth of tunica muscularis fitted a polynomial model. This layer showed a progressive growth from its differentiation to birth. The growth dynamic of serosa was fitted a polynomial type model. This tissue layer displayed a rapid growth from the start of pregnancy to the 20 cm stage, remaining stable to the end of gestation. The abomasum wall grew progressively from the early embryonic stages to birth.

The mathematical models constructed using growth data for each of the stomach compartments closely reflected their prenatal development. This system could thus prove useful for determining fetal age based on the measured thickness of the wall as a whole and of each component tissue layer.



## **H.5 TISSUE RESPONSES AS HEALTH INDICATORS IN THE CLAM *POLYMESODA CAROLINIANA*, FROM TECOLUTLA, VERACRUZ, MEXICO.**

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Ecotoxicological studies in the evaluation of the effects of contaminants is facilitated by the use of bioindicator organisms, which are organisms, characteristic of a system, that allow quantification of the magnitude of stress, habitat characteristics and the degree of exposure of the stressor, or the degree of the ecological response to exposure. Bivalve molluscs have been widely used for this purpose. In Mexico the clams *Polymesoda caroliniana* from Tecolutla, Veracruz, Mexico, system are underutilized resources which also lack ecotoxicological studies. The aim of this study was to make a histopathological study of the mantle, gill and foot tissues of this clam from Tecolutla, Veracruz to show tissue responses. The study was conducted during contrasting climatic seasons (rainy and dry) and different collecting areas. Physicochemical parameters were recorded in the system, 109 clams were collected to assess morphometric and biological parameters. The morphometric data was used to calculate the condition index (Devenport & Chen, 1987). The three organs (foot, gill and mantle) were embedded in paraffin, sectioned (5 µm) and stained in H-E. The histological sections were analyzed by optical microscopy and the prevalence of lesions was evaluated. The recorded physicochemical parameters were within the ranges recommended for the culture and development of organisms; average length was 50 mm and weight of 50 g. The condition index was 2 and 4, which means that they had a moderate level of nutrition. The associated organisms with the clams were annelids and crustaceans. The foot is made up of loose connective tissue and a significant amount of longitudinal and transverse muscle fibers with simple epithelia. Gills are composed of a series of filaments with ciliated cells and basket-like hemolymph lamellae. The mantle is composed of simple epithelium with basal nuclei and loose connective tissue. Tissue responses, for each organ tissue were also observed, which are likely associated with health status, such as: spherical inclusions, lipofuscin granules, vacuolated eosinophil secretions, presence of parasites and inflammation. The biological responses to parasites and inflammations can be regarded as reversible damage. Histopathological studies are an important tool for assessing the health of bivalve molluscs.

### Acknowledgements:

The Biology Master of the Universidad Autónoma Metropolitana Unidad Iztapalapa (UAMI); Indicadores de integridad Ecológica y Salud Ambiental (UAMI); IBEROFUN-CYTED. Incorporación de Nuevos Ingredientes Funcionales a Alimentos como Contribución a la Promoción de la Salud y/o a la Prevención de Enfermedades de la Población Iberoamericana.

## H.6 HISTOMORPHOMETRIC STUDY OF GOAT OMASUM DURING PRENATAL DEVELOPMENT

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Ruminant animals are born with a stomach that is structurally and functionally similar to non-ruminant animals. During the prenatal life, their stomach suffers some morphological changes to suit function in postnatal life. These morphological changes will be associated to the growth and development of gastric viscera.

The aim of this work was to study the growth of each of the tissue layers and the omasum wall by histomorphometric analysis and mathematical models fit growth in order to obtain formulas applicable to embryonic gastric growth.

A total of 140 goat embryos and fetuses, from the first prenatal stages to birth were sampled. Specimens were divided into 5 sequential groups: group I (crown-rump length[CRL] 1.5-4.3 cm, 1-25% gestation), group II (CRL 4.4-8 cm, 25-35% gestation), group III (CRL 9-17.5 cm, 35-50% gestation), group IV (CRL 18-32 cm, 50-75% gestation), and group V (CRL 33-47 cm, 75-100% gestation). Histological sections were viewed through a microscope equipped with a digital video camera. Digital images were analyzed using the Nis-Element 2.30 software package. Variables studied were height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis and serosa) and total wall thickness. One hundred measurements were made in each tissue layer. Tissue growth models were created using four mathematical models: linear, polynomial, logarithmic and exponential. The goodness of fit of each adjustment was measured using the linear coefficient of correlation, *r*. In all cases, embryo body length (in centimetres) was used as the independent variable; the thickness of each tissue layer served as dependent variable.

The epithelial layer growth fitted a polynomial model. The grow-rate was slow from the beginning of the gestation to the 20 cm stage. From this point, a rapid growth was observed to the 30 cm stage; thereafter, the growth-rate was stabilized at the end of gestation. The growth of lamina propria and submucosa fitted a logarithmic model. These layers grew progressively from the early embryonic stages to birth. Tunica muscularis growth was fitted a polynomial model, being recorded a progressive growth from the earliest stages of gestation to birth. The growth of serosa was adjusted to a logarithmic model. Its growth dynamic was characterized by an involution in thickness from the early stages of prenatal development till the end of the gestational period.

The omasum wall grew progressively from the early embryonic stages to birth.

The mathematical models constructed using growth data for each of the stomach compartments closely reflected their prenatal development. This system could thus prove useful for determining fetal age based on the measured thickness of the wall as a whole and of each component tissue layer.

## H.7 HISTOMORPHOMETRIC STUDY OF GOAT RETICULUM DURING PRENATAL DEVELOPMENT

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Ruminant animals are born with a stomach that is structurally and functionally similar to non-ruminant animals. During the prenatal life, their stomach suffers some morphological changes to suit function in postnatal life. These morphological changes will be associated to the growth and development of gastric viscera.

The aim of this work was to study the growth of each of the tissue layers and the reticulum wall by histomorphometric analysis and mathematical models fitted growth in order to obtain formulas applicable to embryonic gastric growth.

A total of 140 goat embryos and fetuses, from the first prenatal stages to birth were sampled. Specimens were divided into 5 sequential groups: group I (crown-rump 1-25% gestation), group II (CRL 4.4-8 cm, 25-35% gestation), group III (CRL 9-17.5 cm, 35-50% gestation), group IV (CRL 18-32 cm, 50-75% gestation), and group V (CRL 33-47 cm, 75-100% gestation). Histological sections were viewed through a microscope equipped with a digital video camera. Digital images were analyzed using the Nis-Element 2.30 software package. Variables studied were height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis and serosa) and total wall thickness. One hundred measurements were made in each tissue layer. Tissue growth models were created using four mathematical models: linear polynomial logarithmic and exponential. The goodness of fit of each adjustment was measured using the linear coefficient of correlation,  $r$ . In all cases, embryo body length (in centimetres) was used as the independent variable; the thickness of each tissue layer served as dependent variable.

The epithelium growth was fitted a polynomial model. Its growth-rate was slow from the earliest embryonic stages to 13.5 cm stage. Thereafter, an active growth was observed, but no continuity to the end of gestation. The lamina propria and submucosa growth was attached to a polynomial model. It was characterized by an involution phase of growth from early embryonic stages to 13.5 cm stage. From this, these tissues showed a rapid growth to the last of gestation. The growth-rate of the muscular tunica was fitted a polynomial model. It continued growing from the early embryonic stages to the end of gestation. The serosa growth fitted a polynomial model. A slow growth was recorded from the start of pregnancy to 17-cm stage; thenceforth, the growth-rate accelerated to birth. The reticulum wall growth followed a polynomial model. Its growth-rate was slow from the early development stages to 13.5 cm stage; thereafter, it showed an accelerated growth to the end of gestation.

The mathematical models constructed using growth data for each of the stomach compartments closely reflected their prenatal development. This system could thus prove useful for determining fetal age based on the measured thickness of the wall as a whole and of each component tissue layer.

## H.8 HISTOMORPHOMETRIC STUDY OF GOAT RUMEN DURING PRENATAL DEVELOPMENT

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Ruminant animals are born with a stomach that is structurally and functionally similar to non-ruminant animals. During the prenatal life, their stomach suffers some morphological changes to suit function in postnatal life. These morphological changes will be associated to the growth and development of gastric viscera.

The aim of this work was to study the growth of each one of the tissue layers and the rumen wall by histomorphometric analysis and mathematical models fitted growth in order to obtain formulas applicable to embryonic gastric growth.

A total of 140 goat embryos and foetuses, from the first prenatal stages to birth were sampled. Specimens were divided into 5 sequential groups: group I (crown-rump length[CRL], 1.5-4.3 cm, 1-25% gestation), group II (CRL 4.4-8 cm, 25-35% gestation), group III (CRL 9-17.5 cm, 35-50% gestation), group IV (CRL 18-32 cm, 50-75% gestation), and group V (CRL 33-47 cm, 75-100% gestation). Histological sections were viewed through a microscope equipped with a digital video camera. Digital images were analyzed using the Nis-Element 2.30 software package. Variables studied were height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis and serosa) and total wall thickness. One hundred measurements were made in each tissue layer. Tissue growth models were created using four mathematical models: linear, polynomial, logarithmic and exponential. The goodness of fit of each adjustment was measured using the linear coefficient of correlation,  $r$ . In all cases, embryo body length (in centimetres) was used as the independent variable; the thickness of each tissue layer served as dependent variable.

The epithelium growth was fitted a polynomial type model. From the earliest stages, epithelium showed an active growth phase which became an involution in the last period of gestation. The growth-rate of lamina propria and submucosa was defined by an involution that began in the early embryonic stages to 20 cm stage. From this stage, a phase of active growth was observed and it continued like that to the end of gestation. Tunica muscularis growth was fitted an exponential model. A slow growth was recorded from the early embryonic stages to 13.5 cm stage; thereafter the growth-rate was increased to the end of gestation. The serosa growth, fitted a polynomial model, was increasing as gestation was progressing. The growth dynamic of ruminal wall was fitted a polynomial model. The growth-rate remained stable from early embryonic stages to 20 cm stage; thereafter, a phase of accelerated growth could be initiated to the final stages of pregnancy.

The mathematical models constructed using growth data for each of the stomach compartments closely reflected their prenatal development. This system could thus prove useful for determining fetal age based on the measured thickness of the wall as a whole and of each component tissue layer.

## **H.9 IMMUNODETECTION OF PRIMARY CILIUM IN THYROID FOLLICULAR CELLS OF DIFFERENT MAMMALS.**

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The primary cilium was first identified in 1898, but it was practically forgotten by the scientific community for many years, as it was considered a vestigial organelle without any known function. Nevertheless, in the last few years, special attention is being paid towards it after the discovery of localized proteins in the cilium that are involved in diverse diseases such as cystic renal disease. Moreover, the presence of primary cilium is considered not to circumscribe to a few cell types but, on the contrary, it is present in numerous cells of mammals, especially in epithelial cells.

The aim of the present work has been to confirm the existence and distribution of primary cilia in human, as well as in pigs, rabbits, guinea pigs, rats and mice thyroid follicles by immunofluorescence using primary cilia specific-antibodies.

Our results confirm an extensive presence of primary cilia in follicular cells of human thyroids. In these cells, the cilium emerges straight, along a perpendicular axis to the apical cell surface and enters the colloid. Furthermore, more than one cilium per cell is observed occasionally. We have also observed the presence of primary cilia in the thyroid follicular epithelium of rabbits, pigs and guinea pigs yet not in rats and mice.

The presence of this ciliary type in thyroid gland of diverse species of mammals as well as its distribution and arrangement on the apical surface of follicular cells, suggests that it could play an important role in relation to thyroid activity that needs to be explored in future research.

## **H.10 CHANGES IN EARTHWORM TEGUMENT WITH AGE: BODY PROTECTION VERSUS REPRODUCTIVE FITNESS**

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Earthworm body wall is highly permeable and responsible for gas and solute exchanges between the soil environment and the animal's internal fluids. Additionally, it provides an effective and protective barrier, aids in locomotion and secretes the structures involved in the mating processes (i.e. secretion of pheromones and formation of the clitellum and tubercula pubertatis).

Since skin changes are among the most visible human signs of aging, variations in the histological structure of invertebrates' epithelium are likely to provide important information about their growing process. Therefore, in this study, we investigated the morphology of the cuticle and the epidermis of the earthworm species *Lumbricus friendi* (Cognetti, 1904) to determine whether their tegument also shows age signs over time.

Mature (clitellum and tubercula fully developed), semi-mature (only tubercula visible) and immature (lacking external sexual characteristics) specimens were collected in the field and taken back to the laboratory. Each individual was then anaesthetized, fixed in an aqueous Bouin's solution and finally embedded in paraffin. Cross sections (5-10  $\mu\text{m}$  in thickness) were stained with hematoxylin-eosin, periodic acid-Schiff (PAS), alcian blue (AB, pH 2.5, pH 1), PAS-AB (pH 2.5) and toluidine blue. Cuticle and epidermis thicknesses were measured in 15-20 random points by microscopy.

Results showed that age had a significant effect on the cuticle and epidermis thickness and whereas the epidermis was significantly thicker in the mature worms than in the semi-mature and juvenile ones, the cuticle followed the opposite trend. These differences were observed in both the anterior part of the body and in the rear one.

Because the cuticle represents the first unspecific barrier of the tegument, it is possible to hypothesise that young specimens will require a greater level of defence against physical and chemical disturbances and pathogens. In contrast, reproductive fitness becomes a more crucial physiological need when reaching maturity.

### **H.11 IMMUNOHISTOCHEMICAL CHARACTERIZATION OF THE ULTIMOBRANCHIAL REMNANTS IN RAT THYROID.**

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Ultimobranchial follicles (UBFs) are considered to be remnants of the ultimobranchial body (UBB). They are structures that vary in size and appearance throughout the "life span" of the rat. According to our studies, in young rats (0-90 days) they appear as "immature follicles", which appear as solid cell nests or tubular structures, whereas the "mature UBFs" predominate in adult rats (6-24 months) with a cystic appearance and lumen with pyknotic nuclei. In addition, in old rats, we have found a few anomalous UBFs which we have termed as "UB cystadenomata".

After a thorough analysis we have detected UBFs in all rat-thyroid samples, although the difficulty to locate them was higher in young rats due to the possibility of mistaking them with normal adjacent follicles. Therefore, the objective of the present work has been to immunohistochemistry characterize these UB remains, with both epithelial differentiation and cellular proliferation markers.

To achieve this aim, Wistar male and female rats of different ages (0-24 months) have been used. After formaldehyde fixation and paraffin embedding, thyroid glands were serially sectioned and stained by immunohistochemistry using the following primary antibodies: anti-Calcitonin (Dako), anti-Thyroglobulin (Dako), anti-Cytokeratins (Z622, A572; Dako), anti-PCNA (Dako) and anti-p63 (Santa Cruz). Immunohistochemical staining has been carried out by using the LSAB+ or ADVANCE kits (Dako. Denmark).

Out of all the markers tested, UBFs were accurately detected with anti-Cytokeratins antibodies, regardless of size and appearance. Thus, we have detected immature UBFs, easily lost among adjacent thyroid follicles, and mature cystic forms besides cystadenomata. Furthermore, we have observed that a high percentage of cells present at the UBFs wall at any age were immunopositive for p63 and PCNA.

Our results demonstrate the possibility of specific UBFs labelling due to their endodermal nature. In addition, we conclude that UBFs maintain their proliferative capacity by expressing specific undifferentiated-cell characteristic markers.

## H.12 TISSUE STRUCTURE AND PARTIAL CHARACTERIZATION OF PROTEOLYTIC ENZYMES OF THREE ORGAN OF THE CLAM *POLYMESODA CAROLINIANA*, FROM TECOLUTLA, VERACRUZ, MEXICO.

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The clam *Polymesoda caroliniana*, or black clam is a characteristic specie from the Gulf of Mexico and highly consumed in the region. The aim of this study was to perform a characterization of the tissue structure, percentage of protein and its proteolytic enzyme activity in 3 organ tissues: mantle, gill and foot. Thirty five clams were collected. The three organ tissues were embedded in paraffin, slides of 5 µm were obtained and stained in H-E. Protein concentration was determined, specific activity and protein content was determined by Kjeldahl's method. Proteolytic activity was calculated at different pH and temperatures and partial characterization of proteins present in the gill via SDS-PAGE were determined. The mantle connective tissue was characterized by simple epithelia and big amounts of mucus; the gills are composed by connective tissue, muscle with a series of filaments and ciliated cells forming breast-like hemolymph lamellae; the foot was composed of connective tissue and a significant amount of transverse and longitudinal muscle fibers with simple epithelia. Gills are responsible for processes such as respiration, selection and movement of food particles. The highest percentage of protein was detected in the foot, an important organ for movement and stimuli reception. The values presented by each organ for protein concentration, percentage and specific activity were respectively, for mantle: 6.1 mg / ml, 86.4 U / mg and 2.2%; gill: 5.2 mg / ml, 126.8 U / mg and 6.6%; and Foot: 5.9 mg / mL, 44.6 U / mg and 7.9%. Proteolytic activity in the gills, showed a range at basic pH and temperatures of up to 70°C. Protein concentration increased in this order: mantle> foot> gill and proteolytic activity was: gill> mantle> foot. Temperature ranges indicated that proteolytic enzymes tolerate high temperature ranges offering a biotechnology opportunity. The biochemical characterization and organ tissues of the clam provides a complete view of the potential of this resource as a functional food and offers complementary techniques that can be of use as a reference for similar studies.

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### **H.13 HISTOLOGICAL STUDIES IN THE OYSTER *CRASSOSTREA VIRGINICA* IN THE GULF OF MEXICO.**

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Environmental monitoring is mostly based on chemical concentrations in the different environmental compartments, including organisms. However, these measurements can't provide information regarding bioavailability and effects of these compounds. Histopathology is a tool that allows the evaluation of tissue changes and has been proposed as a biomarker. Bivalve mollusks have been used internationally in a variety of monitoring programs. The objective of the present work is to present the results of the histological comparison of the oyster *Crassostrea virginica* of five sites: Tecolutla, Alvarado, Tamiahua in Veracruz, Machona in Tabasco and Terminos, Campeche. 158 samples were collected in five sites; these were fixed, sectioned (three serial cuts of 5 µm) and stained with H-E, PAS and Masson. Analyses included observations with optic microscope and the elaboration of a diagnostic table with selection criteria for special histochemical staining. The tissue description emphasized the alimentary channel (esophagus, stomach, stile sac, intestine and digestive gland). The application of special staining techniques, like PAS, demonstrated the type of mucopolysaccharides of the basal membrane in healthy individuals. Collagen presence was recognized and was related to tissue reparation processes. Tissue analysis tables showed lesions prevalence in oysters collected in four out of the five collection sites. Tissue responses included the presence of brown cells, inflammations, parasites, eosinophil secretions, epithelial atrophy of the digestive tubules and the connective tissue, fibrosis and granulomas.

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# **ABSTRACT ADDENDUM**

## **B. 39 ADIPOCYTES FROM HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS AND PRIMARY CILIUM**

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The adipose tissue is really abundant and accessible in human species. Its extraction causes minimal morbidity for the patient and therefore constitutes a suitable source for multipotential mesenchymal stem cells (ADSCs). These cells can be provided from the stromal vascular fraction of the homogenized fat.

Primary cilium is an organelle present in many human cells (1) whose axoneme has 9+0 pairs of microtubules. It projects itself from the cellular surface and has mechanical and chimio-sensorial functions (2).

It is postulated that the primary cilium plays an important role in the potential of differentiation of mesenchymal stem cells (MSCs) (3).

Human ADSCs differentiation process to adipocytes and its relationship with the primary cilium is analyzed in this study.

With this aim three different stages (day 4, 11 and 15) of the *in vitro* ADSC differentiation process were assessed. Immunofluorescent techniques were developed after cellular phenotyping using primary antibodies specific against acetyl- $\beta$ -tubulin and pericentrin in order to recognize the ciliary axoneme and basal body, respectively. An ultrastructural study about the tissue culture corresponding to each of the different stages studied was performed.

The presence of primary cilium was demonstrated in non-differentiated mesenchymal cells as well as in pre-adipocytes (Fig.1).

The role of the primary cilium during the differentiation process and the signaling pathways involved in this process are discussed here.

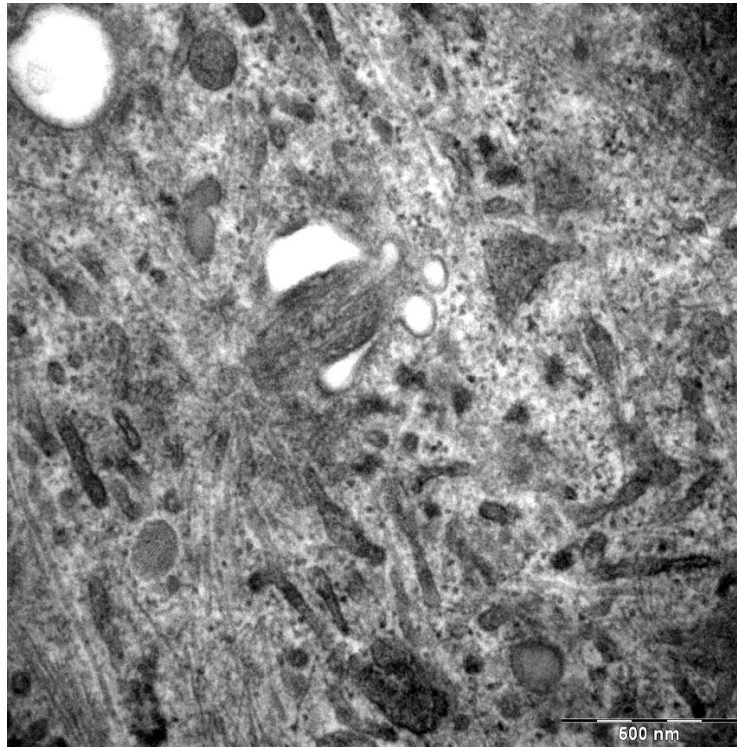


Fig.1.- Primary cilium in *in vitro* ADSCs (15 day differentiation).

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