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# ACTA BIOLOGICA CRACOVIENSIA

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## **Lectures/Invited speakers**



## **IRON IN BIOLOGY: „WHAT IS CALLED A REASON FOR LIVING IS ALSO AN EXCELLENT REASON FOR DYING”**

PAWEŁ LIPIŃSKI\*, RAFAŁ R. STARZYŃSKI, RAFAŁ MAZGAJ, ZUZANNA KOPEĆ

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The ability of iron to readily convert between its two common oxidation states  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  has led to the evolution of several iron-containing protein cofactors that are essential for a wide variety of cellular processes. The redox properties of iron, however, make this metal potentially highly toxic as free iron is a catalyst of oxidative stress via Fenton chemistry. Importantly,  $\text{Fe}^{2+}$  undergoes spontaneous aerobic oxidation to  $\text{Fe}^{3+}$  that is virtually insoluble at physiological pH. This makes the uptake of iron by cells and organisms challenging, despite its high abundance in the environment. Hence, a tight control of iron metabolism is necessary to maintain iron in bioavailable form, to satisfy metabolic requirements for iron, and to prevent excessive iron accumulation. At the systemic level, iron metabolism is regulated by the hepcidin/ferroportin axis that controls iron uptake from the duodenum and reticuloendothelial system, respective sites of iron absorption and recycling. Intracellular iron balance is mainly controlled through iron-regulatory proteins (IRP1 and IRP2) that bind iron-responsive elements in regulated messenger RNAs encoding cellular iron transporters and ferritin, an iron storage protein. Despite such meticulous control, disruptions in iron homeostasis from both iron deficiency and overload account for some of the most common human diseases such as iron deficiency anaemia, hereditary hemochromatosis and disorders that cause ineffective erythropoiesis and secondary iron loading.

### **ACKNOWLEDGEMENTS**

Supported by NCN no. 2019/33/B/NZ9/02566.

## **A JOURNEY THROUGH OVARIAN CELLS DIFFERENTIATION IN RABBITS: FROM GENETIC TO EPIGENETIC MECHANISMS**

NAMYA MELLOUK<sup>1,2,\*</sup>, BÉATRICE MANDON-PÉPIN<sup>1,2</sup>, LUC JOUNEAU<sup>1,2</sup>,  
ANNE FRAMBOURG<sup>1,2</sup>, DOMINIQUE THEPOT<sup>1,2</sup>, AURÉLIE DEWAELE<sup>1,2</sup>, ÉMILIE DUJARDIN<sup>1,2</sup>,  
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In mammals, the formation of the ovary begins early during fetal development by the differentiation of gonadal supporting cells into ovarian pre-granulosa cells. Subsequently, female germ cells differentiate and acquire the ability to enter meiosis. Meiosis initiation is a crucial step for the production of functional haploid gametes. The onset of meiosis is driven by interactions between somatic cells and germ cells and is controlled by a defined transcriptomic program. While this process occurs almost synchronically in the mouse ovary, germ cells enter the pre-meiotic stage between 24- and 28-days post-conception (7 to 3 days before birth) and coexist with proliferating germ cells in the rabbit ovary. The asynchronous transition from mitosis to meiosis results in heterogeneity in the female ovarian cell populations, which makes it challenging to study the mechanisms involved in meiosis initiation and progression. To further understand the process of ovarian differentiation at the level of individual cell populations, we analyzed the transcriptional profiles of single ovarian cells collected from 24 and 28-dpc rabbit fetal ovaries. In addition, we discovered that DMRT1, a conserved transcription factor known to play a crucial role in testis development, is detected in rabbit ovarian germ cells and plays a key role during germ cell differentiation through the regulation of a set of genes involved in meiosis. To further understand the regulatory mechanisms that underly germ cells differentiation we are currently performing a single-cell multiomics analysis (chromatin landscape combined with gene expression) of the developing rabbit ovary.

## **THE LANDSCAPE OF THE IMMUNE SYSTEM IN THE TESTIS AND EPIDIDYMISS**

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Infection and inflammation of the male reproductive tract are significant, and potentially curable, causes of male factor infertility. The defined clinical entities comprise urethritis, prostatitis, seminal vesiculitis, epididymitis, and orchitis. Macrophages comprise the largest immune cell population in the testis and epididymis, organs which are frequently affected.

In both organs, macrophages comprise a heterogeneous immune cell population and display niche-specific phenotypes and functions. This talk will provide information regarding the functions of macrophages during bacterial infections of the testes and epididymis and how understanding macrophage function and macrophage related mechanism of disease in the testis and epididymis may assist in developing new opportunities for intervention in male factor infertility.

# CYTOTOXICITY OF SOME NUTRACEUTICALS AND CERIUM OXIDE NANOPARTICLES ON MCF-7 CANCER CELLS

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Cancer is one of the deadly diseases. Cancer therapy is conducted via chemotherapy, radiotherapy, and surgery as well as alternative ways of traditional medicine. As part of recent developments in nanotechnology, nanoparticulate systems gained significance in medicine. Metal oxide nanoparticles in particular are synthesized as therapeutic agents with promising cytotoxicity in cancer therapy. Cerium oxide (CO) nanoparticles can be used for the treatment of various cancer types and make cancer cells more sensitive to radiotherapy following chemotherapy. However, toxicity of nanoparticles is in discussion. To solve this problem, various approaches such as use of nutraceuticals have been suggested.

In this study, antitumoral activity of CO nanoparticles on breast cancer line (MCF-7) was investigated alone and in the presence of various nutraceuticals (graviola, co-enzyme Q10 and propolis). This was to create synergistic effect and to prevent or reduce toxicity of nanoparticles. Furthermore, antioxidant effect of them was assessed. Also, cytotoxicity of nanoparticles was tested using fibroblast cell line. In this study, CO nanoparticles showed antitumoral activity in time- and dose-dependent manner. The addition of graviola and propolis indicated synergistic effect. On the other hand, they also showed antitumoral activity at certain extent. Fibroblast cell culture study using CO nanoparticles indicated that the toxicity of these nanoparticles is not significant. In addition, tested nutraceuticals showed significant antioxidant activity, which is important in cancer therapy.

In conclusion, cerium oxide nanoparticles can be suggested as effective alternative therapeutic agents for the treatment of breast cancer alone or in combination with nutraceuticals tested in this study. However, *in vivo* studies are still needed to be conducted to support our results.

## ACKNOWLEDGEMENTS

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## **CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY – IN THE SEARCH FOR EFFECTIVE MOLECULAR-BASED THERAPY**

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Chemotherapy-induced peripheral neuropathy (CIPN) is a common dose-limiting adverse effect caused by several antitumor agents, such as platinum derivatives (oxaliplatin and cisplatin), taxanes, vincristine and bortezomib. CIPN affects more than 60% of patients receiving anticancer therapy and it significantly worsens patients' quality of life. The number of analgesic drugs used to relieve pain in CIPN is still limited and their efficacy in CIPN is significantly lower than that in other neuropathic pain types. Despite the increasing knowledge of the etiology of neuropathic pain, this type of chronic pain is resistant to available analgesics in approximately 50% of patients and therefore is continuously a subject of considerable interest for biologists, physiologists, neurologists, medicinal chemists, pharmacologists and other researchers searching for more effective prevention and treatment options for this debilitating condition. Available therapies, as well as results from clinical trials assessing drug candidates for the prevention of oxaliplatin-induced neuropathy are thoroughly investigated, including antagonists of voltage-gated ion channels, antioxidants and neuroprotective compounds. Emerging novel chemical structures as potential future preventative pharmacotherapies for CIPN caused by oxaliplatin, repurposed drugs and combination drug therapy are also considered. Although much is known about molecular mechanisms underlying CIPN, little progress has been made in this area and the analysis of the results of both preclinical and clinical studies shows however that although much is known about potential risk factors and mechanisms underlying CIPN, effective therapies to overcome CIPN are continuously unavailable.

## **ZINC DEFICIENT DIET AS A TRIGGER FOR TREATMENT-RESISTANT DEPRESSION**

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Zinc is an essential element for humans. Its deficiency's negative effects on the human body were first described in 1963. Today, zinc deficiency is believed to affect more than two billion subjects in developing countries. The major symptoms of zinc deficiency are growth retardation, delayed sexual development, and susceptibility to infections. However, zinc deficiency can also lead to depression, increased anxiety, irritability, emotional instability, and induced deficits in social behavior. Several clinical studies have shown that human depression is accompanied by decreased serum zinc levels. Moreover, patients suffering from treatment-resistant depression exhibited much lower serum zinc concentrations than their non-treatment-resistant depressed counterparts. Also, animal studies have shown an important role of dietary zinc deficiency in the induction of depressive-like symptoms. Our recent preclinical results not only confirm these findings but also show that the efficacy of classic monoamine-based or atypical (fast-acting compounds, such as ketamine) antidepressants are blunted under conditions of zinc deficiency in different models of depression. Thus, these results similarly to clinical observations suggest that zinc deficiency may be an important factor in causing drug-resistant depression. Besides, our, as well as other preclinical and clinical data suggest the potential benefits of zinc supplementation as an adjunct to conventional antidepressant drugs.

### **ACKNOWLEDGEMENTS**

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# PROTEOLYSIS IN INFLAMMATION AND IMMUNITY

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Inflammation is a fundamental process in both health and disease. Well-balanced inflammation leads to removal of pathogenic agents and to healing, whereas excessive or prolonged inflammation triggers pathological processes. A current challenge in inflammation research is to find therapeutics that work towards reducing the detrimental properties of inflammation, while enhancing beneficial actions. We explore new strategies to modulate inflammation by studying proteases; key effector enzymes in immune cell function. Our work focusses on understanding which proteases are produced and activated by disease-associated populations of immune cells and how these contribute to inflammation. Given the recent progress in central nervous system macrophage biology and the important functions of microglia in neuroinflammation, we currently focus on macrophages of the central nervous system. In parallel, we also develop new probes and technologies for the detection of active proteases in biological samples and for the modulation of protease activities. This research allows us to obtain a nuanced view on the key proteolytic processes involved in inflammation. By understanding these we will be able to propose innovative strategies to activate anti-inflammatory and pro-repair processes, ultimately leading to novel therapies.

## ACKNOWLEDGEMENTS

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# THE SENSES AND NEURODEGENERATION

MARTIN WITT

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Culinary experiences are conveyed through a range of senses. The multimodal character of the enjoyment of an exquisite Bigos meal results from the interplay of mechanosensory, thermal, visual, gustatory, and olfactory signals. The increasing impairment of these senses in neurodegenerative processes leads to an elementary reduction of the quality of life, but may also be a predictor for worsening of the disease. In particular, the sense of smell is of great importance for diagnosis and therapy due to its distinct dynamic regenerative capacity and its ability to often precede motor and other neurological symptoms.

In this talk, the olfactory system in some common (Parkinson's disease, Huntington's disease, multiple sclerosis) and rare (Niemann-Pick type C1 disease) neurodegenerative diseases will be explained using some animal models. Anatomical, molecular and behavioral patterns are presented.

# **Oral Presentations**

# **PARTICULATE MATTER INDUCES INFLAMMATORY CELL DEATH OF HUMAN MONOCYTES VIA MITOCHONDRIAL AND INFLAMMASOME NLRP3 PATHWAYS**

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Although the efforts to reduce the concentration of particulate matter (PM) in the air of most polluted cities has been recently observed, air pollution remains one of the most harmful factors affecting human's health. The inhaled PM may have different toxic effects depending on its composition, shape and size, its concentration and source. Moreover, PM may be deposited in the lower respiratory tract having an irritating effect, while the smallest may migrate to the blood, affecting the functions of circulating leukocytes. Here, we asked whether PM has impact on the functions of peripheral blood monocytes.

Monocytes isolated from peripheral blood of healthy donors were exposed *in vitro* to two types of PM (NIST; standard urban particulate matter or LAP; PM without the organic fraction) used in different concentrations.

After the PM treatment a significant reduction of the antigen-presenting capacity of monocytes, alterations of their morphology, decrease in cell viability and increase of pro-inflammatory cytokine production were observed. Moreover, the obtained data showed changes in monocyte oxygen metabolism, enhanced ROS production, decrease of mitochondrial membrane potential and activation of Caspases 9 and 3, which were mostly dependent on the inorganic components of air pollution. The activation of inflammasome NLRP3 and Caspase 1, followed by the secretion of IL-1 $\beta$  was observed in parallel, however these were more dependent on the organic fraction of PM.

These observations suggest a new role of PM in cell death, depending on the simultaneous activation of two signaling pathways (mitochondrial and inflammasome NLRP3), leading to strong inflammatory reaction.

# **BISPHENOL S AND BISPHENOL F STIMULATE PROLIFERATION AND MIGRATION OF ADULT GRANULOSA CELL TUMOR**

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Bisphenol S (BPS) and bisphenol F (BPF) are endocrine disrupting molecules that are used in plastics. Because of their widespread use, these compounds have been detected in human urine and follicular fluid. Therefore, hormone-sensitive cancers, such as ovarian cancers, are exposed to these compounds. In the present study, our objective was to determine the effects of BPS and BPF on the proliferation and migration of the human ovarian adult granulosa cell tumor line (KGN).

In this study, we have found that BPS (100 nM) and BPF (0.1 nM, 1 nM and 10 nM) increased KGN cells proliferation. Furthermore, using a wound healing assay, we demonstrated that BPS and BPF in a statistically significant manner stimulated KGN cell migration. Furthermore, we found that neither bisphenol affected MMP-2 expression, but both increased its activity. Of the genes that mediate EMT, only *CLDN3* expression was found to be upregulated by BPF treatment, but this is of interest because it is one of the most upregulated genes in ovarian cancer.

In summary, BPS and BPF induce proliferation and migration that contributed to the progression of adult granulosa cell tumor progression.

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**IMPACT OF DISTURBANCES IN COPPER METABOLISM  
ON THE COURSE OF SPERMATOGENESIS IN MALES WITH MUTATIONS  
IN THE *ATP7B* GENE - A MOUSE MODEL OF WILSON'S DISEASE**

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Copper is a trace element necessary for the growth and development of all living organisms. Due to the redox properties, this metal is a cofactor of many enzymes involved in fundamental metabolic processes. However, redox properties can contribute to the production of free radicals in the Fenton reaction. Copper also plays a key role in the male gonads during spermatogenesis when male gametes are produced. It is well known that testes are very sensitive to both copper deficiency and overload. For instance, an appropriate copper level is necessary to initiate meiosis in premeiotic germ cells. Furthermore, sperms, due to their movement ability, contain many mitochondria in the midpiece. It is perfectly known that copper is a cofactor of cytochrome c oxidase indirectly responsible for energy production, which can be related to the copper influence on sperm movement ability. The balance between essential and harmful copper concentrations in male germ cells and in Sertoli cells that support their development is pivotal.

In the present study, we investigated copper contents in mice' testes, testis histology and mass, and sperm quality by using sperm tests. The analyses were conducted on *tx-J* mutants (an animal model of Wilson's disease) and wild-type 3- and 6-month-old mice. We observed differences in copper contents in testes, testis histology and mass, and sperm motility between wild-type and mutant mice in respective age groups. Our results may suggest higher Cu concentration in testes can negatively impact male fertility.

## ACKNOWLEDGEMENTS

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# **ROLE OF GHRELIN AND IGF-1 IN GASTROINTESTINAL DEVELOPMENT IN NEWLY HATCHED CHICKENS**

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After hatching significant changes occur in the anatomy and physiology roles of the digestive system. The feeding behavior of chicken is regulated by several peptides, one of which is ghrelin. Ghrelin is a feeding-promoting peptide in mammals, opposite to avian species where presumably inhibits the appetite. Ghrelin in avian species affects the growth, development, and motility of the digestive system. Ghrelin is involved in the regulation of energy homeostasis and its administration may increase body weight gain and food intake. Insulin-like growth factor-1 (IGF-1) is also the regulator of metabolism in chickens. Understanding the regulatory mechanism of feed intake in chickens is important as metabolic diseases in broiler and laying chickens are serious problems in the poultry industry.

Due to various anatomy of the digestive tract, the question arises about the role of ghrelin and IGF-1 in each part of the gastrointestinal tract of chickens. In spite of extended research, there is still a lack of information about the degree of ghrelin and IGF-1 secretion from the crop, stomach, and intestine in intact chickens i.e. without interaction with other peptides.

The aim of the study was to investigate the changes of ghrelin and IGF-1 concentration in the crop, stomach, and intestine of newly hatched chickens. The experiment was carried out on newly hatched chickens (n=24), 2 (day 0) and 24 hours (day 1) after hatching. Ghrelin and IGF-1 levels were measured by RIA method.

The highest concentration of ghrelin was found in the stomach in day 0 and in the crop in day 1. The highest concentration of IGF-1 was in the duodenum in day 0 and day 1.

The obtained results suggest that ghrelin and IGF-1 might be the crucial stimulators of growth and development as well as modulators of GI tissues functions in growing hens.

## **ACKNOWLEDGEMENTS**

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# TIME-COURSE CHANGES IN IRON CONTENT AND TISSULAR DISTRIBUTION AFTER *PER OS* SUCROSOMIAL FERRIC PYROPHOSPHATE (SIDERAL®) ADMINISTRATION TO -REPLETE AND IRON-DEFICIENT 14-DAY-OLD PIGLETS

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**Introduction:** Iron dextran injection is a common treatment to cure iron deficiency anemia (IDA), but can lead to health problems. We monitor time-course changes of iron distribution after *per os* dosage (10 mg Fe/kg b.w) of SiderAL® (SI), to iron-replete (Fe-rep) and iron-deficient (Fe-def) 14-day-old piglets. Samples from 3 piglets per group were collected at: 0, 6, 12, and 24 hours.

**Results:** RBC indices tightly reflected normal and anemic initial (time-point 0) status of Fe-rep and Fe-def animals. The consistently depressed serum iron levels was observed in Fe-def compared to Fe-rep animals. Hcpidin mRNA expression in liver showed significant increase in Fe-rep piglets at time point 6. Bone marrow mRNA ERFE showed significant increase in Fe-def piglets. Non-heme iron content in tissues showed the differences between Fe-rep and Fe-def piglets at the time-point 0 and in successive time points. Iron staining showed duodenal and bone marrow accumulation of iron.

**Conclusions:** A single dosage of SI can enhance erythropoiesis. In the time point 24 plasma iron level decreased in both groups with a tendency to maintain higher level in Fe-rep piglets. SI has a potential to induce hepcidin expression only in Fe-rep piglets. Microscopic analysis of liver sections showed iron deposits of Fe-rep piglets at the time-point 0 and 24, and no evidence that administration of SI influenced the intensity of iron staining and the distribution in the liver. The stainable iron in bone marrow of Fe-def piglets proves that SI-derived iron is rapidly utilized for erythropoiesis.

## ACKNOWLEDGEMENTS

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**CELLULAR ORIGIN OF MICROPARTICLES  
DURING SYSTEMIC INFLAMMATION IN MICE –  
IN VIVO AND EX VIVO STUDIES**

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Microparticles (MPs) are essential for cell-cell communication both in homeostasis and pathological conditions. They carry/transport multiple bioactive cargo over long distances, including proteins, lipids and genetic materials. Their presence and activity can be detected in various body compartments. During inflammation, neutrophils secrete MPs but also eject neutrophil extracellular traps (NETs). Both structures impact the course of inflammation and can interact with each other. Therefore, the aim of the study was to investigate MPs origin and correlate it with NETs during systemic inflammation in mice (healthy *vs.* endotoxemic). *Ex vivo*, MPs were analyzed by flow cytometry and Nanoparticle Tracking Analysis (NTA) in two types of body fluids – blood and peritoneal exudate, and the analysis indicated low counts of MPs during homeostasis and their increased numbers upon endotoxemia. *Ex vivo*, the MPs obtained from the circulation and peritoneal fluids were of platelet and leukocyte origin. *In vivo*, MPs were visualized with intravital microscopy (IVM) in liver sinusoids (vasculature) and they were dominantly of neutrophil- and monocyte/macrophage-origin. Amounts of leukocyte-derived MPs and released NETs increased during systemic inflammation and the former were present in NETs. The studies confirm heterogeneity of MPs and strongly indicate an interplay between the two types of released structures, but further studies are required to verify if this crosstalk is beneficial or pathological for the sepsis outcome.

#### ACKNOWLEDGEMENTS

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## **PHENOTYPIC, FUNCTIONAL AND GENETIC CHARACTERIZATION OF NORMAL DENSITY NEUTROPHILS AND PMN-MDSC IN PATIENTS WITH COLORECTAL CANCER**

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Almost a century ago it was shown that cancer development is accompanied by neutrophilia, however so far, the role of neutrophils in cancer has not been fully elucidated. Based on the density-gradient centrifugation of peripheral blood, circulating neutrophils in cancer patients may locate in two fractions – the interphase, where the so-called low-density granulocytes (containing myeloid-derived suppressor cells of granulocyte origin - PMN-MDSCs), co-localize with peripheral blood mononuclear cells; and - in the pellet, where locate the residual neutrophils with normal density (NDNs). However, differences between these populations in cancer patients has not been yet fully determined.

In our study we analyzed phenotypic, functional, and genetic characterization of both neutrophil subsets in patients with colorectal cancer (CRC) and compared to healthy donors (HD). The results showed significantly higher expression of PD-L1, VISTA, LOX-1, and ARG-1 on PMN-MDSCs than NDNs in patients, however, these populations did not differ in their ability to suppress mitogen-driven proliferation of autologous T lymphocytes *in vitro*. Further, to deeply characterize both subsets, a microarray gene expression analysis was performed. This analysis revealed approximately 350 genes overexpressed in NDNs and PMN-MDSCs from CRC, when compared to NDN from HD. Among the upregulated genes there were ones involved in the inositol phosphate metabolism, lysozyme activity and reactive oxygen species-dependent pathways. It seems that in CRC patients the whole population of circulating neutrophils present characteristics related to their suppressive activity.

# RED BLOOD CELLS AS IMMUNE SENTINELS – IMPACT ON NEUTROPHIL ACTIVITY IN HOMEOSTASIS AND SYSTEMIC INFLAMMATION

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Red blood cells (RBC) or erythrocytes for decades were considered to function as inert oxygen transporters filled with hemoglobin, however, recently they started to be recognized as “immunologically savvy”. This is because apart from their traditional functions, RBCs can bind pathogens, store cytokines and catch DNA from microbial invaders or damaged tissues alerting the immune system of danger. Enriching these findings, herein we show that RBCs (in both *ex vivo* and *in vivo* conditions) interact with leukocytes, especially neutrophils, and modulate their activity. In particular, we scrutinized impact of freshly isolated RBCs on neutrophil metabolic and immunological parameters upon their co-incubation (murine cells) and we show that RBCs prolonged neutrophil survival but diminished the release of neutrophil extracellular traps (NETs). We further studied interactions of RBCs with leukocytes and platelets upon *in vivo* conditions during homeostasis and bacterial sepsis with intravital microscopy (IVM). In mice endotoxemia was induced with lipopolysaccharide (LPS) and the cells were imaged in the liver vasculature (sinusoids) upon their staining with flurochrome-conjugated monoclonal antibodies. The obtained results indicate that RBC interact with neutrophils, macrophages and platelets more frequently during sepsis in comparison to healthy individuals. This was visualized on recorded videos as well as when performing 3D z-stacks and analyzing them by a semi-transparent mode (Imaris). We conclude that erythrocytes have a diverse modulatory effect on functioning of neutrophils depending on local milieu and they appear to impact immune responses also *in vivo*.

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# SHORT-TERM BLUE LIGHT EXPOSURE AFFECTS BRAIN FUNCTIONING OF *DROSOPHILA MELANOGASTER*

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The impact of the blue light on the nervous system is still poorly understood, though smartphones, TVs and computers are providing this type of radiation every day. Hence, it is of great importance to determine how exposure to blue light affects physiology of nervous system. The research regarding this topic conducted on *Drosophila melanogaster* revealed that the long-term exposure to the blue light results in reduced lifespan, motor impairment and altered expression of genes related to oxidative stress. In our project we used the following strains of fruit fly as the research object: Canton-S (wild type),  $w^{1118}$  (white-eyed mutants), *park* mutants (Parkinson's disease model) to test whether the short-term exposure to the blue light in the evening impacts brain functioning. We observed changes in expression level of genes related to oxidative stress, dopamine pathway and autophagy. Additionally, our experimental conditions affected sleep level and pattern. Obtained results varied in an age- and genotype-dependent manner, which implies that the blue light may be harmful especially to individuals with Parkinson's disease. To explain this phenomenon we characterized dopaminergic neurons in the second optic neuropile, which could be the link between light pollution, sleep disruptions and Parkinson's disease. Our data provide new insight on the role of light on the progression of neurodegenerative disorders.

## ACKNOWLEDGEMENTS

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# **Poster Presentations**

## **CELL CYCLE GENES IN JUVENILE OVARIAN GRANULOSA TUMOR: STEPS TOWARD EARLIER DIAGNOSIS AND NOVEL THERAPIES**

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The etiology of juvenile ovarian granulosa tumor (JGCT) remains unclear. Moreover, its extreme rarity represents a limitation in our understanding of its management and prognosis. On the other hand, human malignant tumors are characterized by abnormal proliferation resulting from alterations in the regulatory mechanisms of the cell cycle. In the present study, our aim was to describe abnormalities in the expression of cell cycle regulator genes in the JGCT cell line.

The human granulosa cell line HGrC1 and the juvenile granulosa tumor cell line COV343 have been used as *in vitro* models. To examine relative expression (RQ) in the two cell lines, expression in HGrC1 was set as 1. In COV343 cells, the basal expression of the cell cycle regulator genes *CDK2* (25-fold), *CCNE1* (45-fold), *CCNA2* (5-fold), and *CCKN1A* (8-fold) was higher than in HGrC1 cells. Whereas the expression of *CDK4*, *CCND1*, *CCND2*, and *CCND3* was similar in both cell lines.

In summary, these findings demonstrate that cell cycle regulatory genes such as G<sub>1</sub>-S, and S could play a crucial role in the development of JGCT. Additionally, they could be valuable markers for the development of more targeted therapies.

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## THE EFFECT OF $\Delta$ M3 AND $\Delta$ M4 SYNTHETIC PEPTIDES ON LUNG CANCER CELL LINE A549

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A new approach in cancer biochemistry concerns the use of antimicrobial peptides as chemotherapeutic agents. In this study we used synthetic M3 and M4 peptides to evaluate its cytotoxic properties in the A549 human epithelial lung cancer cell line. In addition, an attempt was made to determine the mechanism of action of these peptides. Cell viability was verified by AlamarBlue cytotoxic assay. Cell membrane permeabilization was examined by cSyttox and lactate dehydrogenase (LDH) tests. Reactive oxygen species (ROS) were measured by nitro-blue tetrazolium (NBT) assay. Selected apoptosis markers were assessed, including caspase 3/7 activity with Caspase-Glo assay and Bax and Bcl-xl protein expression by Western Blotting. The wound healing assay was used to determine the impact of peptides on cell migration.

A significant decrease in cell viability after 24h with M4 was observed (IC<sub>50</sub> = 12.5  $\mu$ M), whereas concentration above 25  $\mu$ M led to a complete loss of viability. At the same concentrations,  $\Delta$ M3 was not cytotoxic or caused a weak reduced viability.  $\Delta$ M4 was more effective in destabilizing the cell membrane as compared to  $\Delta$ M3. Moreover, this peptide caused an increased ROS production, suggesting the mediation of oxidative stress in its toxicity. Both peptides inhibited cell migration and disturbed the expression of pro- and anti-apoptotic proteins. The above observations indicate that the tested peptides show promising potential in the fight against cancer cells and are promising candidates for further research on finding new drugs for targeted therapy in cancer.

**ITACONIC ACID SUPPRESSES FORMATION  
OF NEUTROPHIL EXTRACELLULAR TRAPS (NETs)  
VIA IMPACT ON HYPOXIA INDUCIBLE FACTOR (HIF-1 $\alpha$ ),  
NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 (NRF2)  
AND HEME OXYGENASE (HO-1) EXPRESSION**

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Itaconic acid is an anti-microbial intermediate produced as a consequence of the first break in the Krebs cycle. The second break in this cycle leads to activation of hypoxia-inducible factor-1 $\alpha$  (Hif-1 $\alpha$ ) – the regulator of response to hypoxia. Previous studies showed that derivative of itaconate – 4-octyl itaconate (4-OI) activates transcription of the anti-inflammatory nuclear factor erythroid 2-related factor 2 (Nrf2) and increase protein expression of cyto-protective heme oxygenase (HO-1), while its effects on neutrophil effector functions are not known. For this reason, the aim of the current study was to determine effects of itaconic acid on neutrophil extracellular traps (NETs) formation in relation to Nrf2, HO-1 and Hif-1 $\alpha$  expression. NETs are beneficial at the early stages of infection but later on they are detrimental to the host cells. For this reason, it is crucial to identify selective inhibitors by which NET formation could be controlled. In the study, we tested different parameters of bone marrow derived neutrophils isolated from mice, both lean and obese (on high-fat diet) as well as originating from aged animals. Neutrophils were treated with 4-OI and/or inhibitors of metabolic pathways (HO-1/Hif-1 $\alpha$ ) in the presence or absence of lipopolysaccharide (LPS). We report that 4-OI dramatically decreased formation of NETs by LPS stimulated cells independently of the age (young/old mice) and metabolic state (lean/obese mice) of animals from which they were isolated, and this effect was mediated via induction of Nrf2/HO-1 and Hif-1 $\alpha$  inhibition.

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# CAN REPLICATION STRESS AND DNA DAMAGE BE REGULATED BY HEME OXYGENASE-1?

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Heme oxygenase-1 (Hmox1) degrades heme and reduces both hemolytic and oxidative stress. Our previous studies have shown that Hmox1-deficient cells are characterized by elevated levels of G-quadruplexes (G4). G4 structures are three-dimensional structures of nucleic acids that can form on single-stranded DNA in the replication forks. As a spatial hindrance, G4 can inhibit polymerase progression, leading to replication stress. If stalled forks are not unwind, they eventually collapse, causing double-strand DNA breaks. Being aware that heme stabilizes G4 structures, we investigated whether Hmox1 plays a role in preventing replication stress. Using the DNA Fibers Assay, we found a higher number of stalled replication forks in the Hmox1-deficient cells cultured in vitro. Next, we confirmed that this effect is potentiated by increased heme synthesis, after treatment of cells with  $\delta$ -aminolevulinic acid (ALA), and an elevation of endogenous free heme. Inhibition of replication reduced the rate of cell proliferation and was accompanied by enhanced DNA damage. On the other hand, exogenous hemin led to oxidative stress and boosted the DNA repair response. Thus, an excess of exogenous and endogenous heme leads to distinct effects: oxidative stress or replication stress, respectively. Finally, the replication stress in Hmox-1 deficient cells led to a reduced rate of cell proliferation and upregulation of type-I interferon response. In summary, Hmox1 protects cells from replication stress induced by free heme, a G4 stabilizer.

## ACKNOWLEDGEMENTS

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# **STUDIES ON MONOCYTES: ESTABLISHING A PROTOCOL FOR IMMUNOMAGNETIC ISOLATION OF MONOCYTES AND IDENTIFICATION OF THEIR MARKERS**

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Monocytes play key roles in inflammation and can be divided into two different subsets: classical (proinflammatory; CCR2<sup>+</sup>) and nonclassical (alternative/anti-inflammatory; CX3CR1<sup>+</sup>) monocytes. Despite availability of various protocols for monocyte isolation such as their separation by Percoll/Histopaque gradient centrifugation and subsequent isolation based on adherence to culture dishes, sorting of highly pure monocytes suitable for further applications remains a challenge. Moreover, specific identification of monocytes is not fully optimized. Knowing that mouse monocytes commonly express CD115 and F4/80 surface antigens, first we evaluated a method of immunomagnetic isolation of bone marrow-derived monocytes using a commercial kit (Miltenyi Biotec) where non-target cells were depleted - retained in separation columns by magnetic field - while unlabeled monocytes passed through columns. Next, the purified monocytes were analyzed using flow cytometry revealing that nearly 90% were F4/80<sup>+</sup>, 69% were CD115<sup>+</sup> and only 2% of them were Ly6G<sup>+</sup> (a neutrophil marker) confirming high purity of monocytes. Interestingly, only 45% of monocytes were double positive for CD115<sup>+</sup> and F4/80<sup>+</sup>. Furthermore, we investigated monocyte markers *in vivo* in intact and septic mice using intravital microscopy. In intact and septic mice we observed monocytes positive for F4/80, CD115 or double positive monocytes (F4/80<sup>+</sup> CD115<sup>+</sup>). However, the changes in proportion and number of monocytes with expression of these markers need to be further investigated.

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# RESISTIN AFFECTS LUTEOLYSIS IN THE PORCINE CORPUS LUTEUM VIA REGULATION OF AUTOPHAGY

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Corpus luteum (CL) function is production of progesterone (P4), which prepares the uterus for implantation. When fertilization does not occur luteolysis is mediated by apoptosis or autophagy and a new cycle may begin. Nevertheless, reduction in the luteal phase leads to implantation problems and pregnancy loss, so this process is strictly controlled by the endocrine system. Our previous studies showed that adipokine - resistin decreases P4 level in porcine CL, which is one of the features of luteolysis and stimulates apoptosis of luteal cells. Hence, the aim of the present study was to investigate the effect of resistin on luteal cells autophagy.

Luteal cells were isolated at the middle luteal phase. After 24h of luteal cells preincubation resistin at physiological dose 1 ng/mL was added to 24, 48 and 72h. Then, autophagy markers: beclin-1, microtubule associated protein (LC3) and lysosomal-associated membrane protein 1 (LAMP1) mRNA and protein expression were analyzed by real-time PCR and Western blot, respectively. Statistical analyses were performed by Graph Pad Prism 5 software using a one-way ANOVA.

Our data showed that resistin in the time-dependent manner enhanced luteal cells autophagy: increased beclin-1 and LC3 transcript and protein expression after 24 and 48h of *in vitro* culture. However, this adipokine did not affect LAMP1 level ( $n=6$ ,  $p<0.05$ ).

Results indicated the participation of resistin in luteal cells regression by direct changes in autophagy. Taken together, resistin by influence on CL luteolysis may be a risk factor for female luteal cells function and consequently fertility.

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# **EFFECT OF EICOSAPENTAENOIC ACID AND METFORMIN ON THE LEVEL OF PRO-INFLAMMATORY GENES AND PROTEINS IN MURINE ADIPOCYTES**

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Polyunsaturated fatty acids (PUFAs) are involved in many critical biological processes, including eicosanoid synthesis, cell signaling, inflammation, and the regulation of gene expression.

The study aimed to evaluate the effects of eicosapentaenoic acid (EPA) and metformin (MET) on the expression of pro-inflammatory genes and proteins in murine adipocytes activated with lipopolysaccharide (LPS).

A significant decrease in pro-inflammatory proteins TLR4, cPGES, and COX-2 in inflamed murine adipocytes was observed after EPA and MET treatment. It has also been shown that in 3T3-L1 cells, after supplementation with EPA+MET and LPS activation, there was a significant decrease in the expression of pro-inflammatory genes.

The results confirm that EPA and MET, through their synergistic anti-inflammatory action, have a beneficial effect on adipocytes.

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# THE EFFECT OF VISFATIN ON THE EXPRESSION OF SELECTED CELL CYCLE PROTEINS IN HUMAN PLACENTAL CELLS

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Placentation is a dynamic and complicated process; after implantation, trophoblasts differentiate into cyto- and syncytiotrophoblast cells, and proliferate much more rapidly than embryo cells, creating an organ in just a few weeks that properly fulfils its function and supports foetus development. Placentation requires the production of growth factors, adhesion proteins, hormones and transcription factors. Visfatin as a one of adipokines plays pleiotropic functions in human organism including regulation of energy metabolism, immune, cardiovascular or even reproductive system processes. The aim of this study was to investigate the effect of visfatin on key cell cycle factors level, including proliferating cell nuclear antigen (PCNA) and cyclins in placenta cell line JEG-3.

*In vitro* cell culture of JEG-3 was conducted in DMEM/ F12 with 1% FBS supplemented with 10 ng/ ml of visfatin for 48 and 72h. Transcript and protein levels of PCNA and cyclins D, E, A, B were measured by real time PCR and Western blot, respectively (n=4). Statistical analysis was performed in GraphPad Prism 8 using one - way ANOVA and Tukey's tests (p<0.05).

We observed that visfatin in time- dependent manner decreases significantly the transcript and protein levels of PCNA and cyclins D, A, E, B in JEG-3 cells which emphasizes its reducing effect on cell cycle progression. Obtained results showed that visfatin decreases placental cell proliferation and suggest its regulate role in the placenta physiology.

## ACKNOWLEDGEMENTS

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**RED BLOOD CELL LYSATE ACTIVATES  
LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGES  
TO OVERPRODUCE NITRIC OXIDE (NO) VIA UP-REGULATION  
OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS)**

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Abnormal disintegration of red blood cells (RBC), also known as hemolysis, results in extracellular release of hemoglobin and other cellular components. Until now, effects of a complete hemolysate on expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) by macrophages have not been studied in detail. Thus the aim of the current study was to determine impact of hemolysate obtained from freshly isolated erythrocytes on the immune activity of murine macrophages (of RAW 264.7 cell line). The cells were treated with various concentrations of the hemolysate (originating from  $2,5 \cdot 10^6$  or  $25 \cdot 10^6$  RBC) collected from whole blood murine samples (C57Bl/6J mice), in presence or absence of lipopolysaccharide (LPS). Macrophage viability, mitochondrial activity, NO production and iNOS expression were evaluated. It turned out that both hemolysate concentrations impacted macrophage effector functions but in a concentration-dependent manner. The lower concentration induced some macrophage cytotoxicity and down-regulated their mitochondrial capacity but had no impact on NO synthesis. Whereas the higher concentration increased macrophages survival, and possibly even induced their proliferation, but only when LPS was additionally present. High concentration of hemolysate alone did not alter macrophage viability or NO release, however, it did lower cell metabolism. When macrophages were stimulated with the higher concentration of hemolysate, not only did they produce more NO but this was due to up-regulation of iNOS expression. Therefore whereas mild hemolysis is rather detrimental to macrophages, a stronger RBC lysis activates the cells towards a profound pro-inflammatory phenotype, especially if inflammatory factors are already present in the milieu.

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# HISTOLOGICAL AND HISTOCHEMICAL ANALYSIS OF THE SPLEEN FROM THE OLD HEME OXYGENASE 1 DEFICIENT MICE

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Iron (Fe) is a microelement indispensable for normal growth and development for near all living organisms. Due to its redox ability iron play an important role as a cofactor of many enzymatic proteins required to sustain fundamental life processes, including DNA synthesis and repair, ATP production, cell cycle, oxygen transport, and detoxification. Heme oxygenase 1 (HO1), coded by the *Hmox1* gene, is an inducible, stress-responsive, multifunctional enzyme playing an important role in inflammation and iron homeostasis. It catabolizes free heme into ferrous iron ( $\text{Fe}^{2+}$ ), carbon monoxide (CO) and biliverdin converted to bilirubin. HO1 is highly expressed in the macrophages and is a key protein for iron recycling from senescent red blood cells and therefore play a major role in controlling the bioavailability of iron for erythropoiesis. In the *Hmox1*<sup>-/-</sup> knockout mice, HO1 deficiency results in disturbances in iron reutilization associated with the inability of macrophages to catabolize heme and their progressive loss during the lifespan caused by toxic effect of heme. *Hmox1*<sup>-/-</sup> knockout mice progressively developed intravascular haemolysis with age and iron-deficiency anaemia because in HO1 deficient mice iron is trapped in the kidney and liver. All pathological symptoms increased with the age of *Hmox1*<sup>-/-</sup> knockouts. Results of histological analysis revealed that in the spleen of the knockout mice elimination of red pulp macrophages started early, even in young 3-month-old individuals, pathological changes increase with age and in 24-month-old mice leading to spleen atrophy caused by fibrosis and necrosis of the red and white pulp.

## ACKNOWLEDGEMENTS

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# **THE USE OF SIZE EXCLUSION CHROMATOGRAPHY (SEC) FOR THE ISOLATION OF EXTRACELLULAR VESICLES FROM PORCINE OVARIAN FOLLICLES**

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Extracellular vesicles (EVs) are membrane-bound nanoparticles that are released by different cell types and play a crucial role in the intercellular communication. They carry an active biological cargo such as DNA, RNA, proteins and lipids. This EVs characteristic gives the possibility of their practical use for diagnostic and therapeutic purposes. The presence of EVs has been demonstrated in the mammalian ovarian follicle, but their role is not fully described. Given that EVs are a new element of communication within the ovarian follicle, the extensive research is needed to optimize method of their effective isolation. Size exclusion chromatography (SEC) is one of the best practices for EVs isolation/purification as it recovers a sufficient amount of EVs retaining their functionality. In this study, we assessed the quality and efficiency of EVs isolation from follicular fluid (FF) of porcine antral follicles using SEC. FF samples were loaded on a sepharose columns, 20 fractions were collected and analyzed by nanoparticle tracking analysis, transmission electron microscopy and Western blot analysis for the expression of EVs markers (CD9, CD63). Our results show that SEC is an effective method of EVs isolation from porcine FF maintaining they structure. This method allow to remove most of the abundant FF proteins and use EVs for functional analyses.

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**IMPACT OF LITTER SIZE ON HEMATOLOGICAL AND IRON STATUS OF SOWS  
AND NEWBORN PIGLETS: A COMPARATIVE STUDY ON DOMESTIC PIGS  
WITH THE VARIED NUMBER OF PIGLETS IN THE LITTER  
AND WILD BOARS**

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Iron deficiency anemia (IDA) is the most prevalent deficiency disorder in neonatal period in domestic pigs (*Sus scrofa domestica*). The primary cause of IDA in suckling piglets is critically low hepatic iron reserves. Large litter size has remained one of the main objectives of high pressure in pig breeding applied through the last century and very likely contributed to the paucity of iron stores. The aim of this study was to check if the varied number of piglets in the litters of Large Polish White (LPW) sows influences red blood cell (RBC) indices and blood plasma iron parameters in sows after farrowing and their newborn piglets. We used animals from small and large size litters obtained by regulated embryo transfer as well as animals from naturally size litters. Considering that no cases of iron deficiency have been reported in the offspring of wild boar (*Sus scrofa*), the ancestor of domesticated pigs, having usually less piglets in the litter as domestic pig. The next goal of our study was to compare RBC and iron status between wild boars and LPW pigs from a varied size litters. Our results challenge the established view that the litter size in the domestic pig determines RBC and iron status in newborn piglets. To our knowledge, we showed for the first time the experimental evidence that RBC and iron status in wild boar sows and piglets are higher than in domestic pig animals regardless of their litter size.

#### ACKNOWLEDGEMENTS

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**EFFECT OF POLYCHLORINATED BIPHENYLS (PCB118 AND PCB153)  
AND THEIR HYDROXYLATED METABOLITES (4-OH-PCB107  
AND 3-OH-PCB153) ON BASAL AND DEX-MODIFIED T<sub>3</sub> SECRETION  
FROM CHICKEN LIVER EXPLANTS**

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With industrialization, the production of chemicals and their introduction into the environment has increased massively. Some of these chemicals function as endocrine disruptors (EDs), and this group includes polychlorinated biphenyls (PCBs) and their hydroxylated metabolites (OH-PCBs). To assess the *in vitro* effect of PCBs and OH-PCBs on triiodothyronine (T<sub>3</sub>) secretion from the chicken liver, explants of this tissue were incubated for 6 h in medium supplemented with dexamethasone (DEX) (100 nM/ml), PCB118 (dioxin-like PCB), PCB153 (non-dioxin-like PCB), 4-OH-PCB107, and 3-OH-PCB153 ( $0.5 \times 10^{-8}$  M), and DEX together with each PCBs and OH-PCBs. Concentration of T<sub>3</sub> were determined by RIA method. The results obtained were statistically evaluated by means of one-way analysis of variance (ANOVA); differences between means were analysed by post-hoc Tukey's test at  $p < 0.05$ . The results of the *in vitro* experiment revealed that, both PCB congeners, PCB118 and PCB153, decreased the concentration of T<sub>3</sub> in the medium. Only, 3-OH-PCB153 increased the secretion of this iodothyronine. None of the tested PCBs and OH-PCBs influenced the DEX-inhibited secretion of T<sub>3</sub> from liver explants. However, compared to the control group, DEX abolished the stimulating effect of 3-OH-PCB153. The results obtained revealed that the tested PCBs and OH-PCBs interacted with and/or abolished the inhibitory effects of DEX on T<sub>3</sub> secretion. Moreover, these results indicate that not only parental PCBs but also their hydroxylated derivatives may influence iodothyronine metabolism in the chicken liver, resulting in changes in T<sub>3</sub> availability in the organism.

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## **THE SENSE OF SMELL IN AN ANIMAL MODEL OF HUNTINGTON´S DISEASE**

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For neurodegenerative diseases such as Huntington's disease (HD), early diagnosis is essential to treat patients and delay symptoms. Impaired olfaction, as observed as an early symptom in Parkinson's disease, may also constitute a key symptom in HD. However, there are few reports on olfactory deficits in HD. Therefore, we aimed to investigate, in a transgenic rat model of HD: (1) whether general olfactory impairment exists and (2) whether there are disease-specific dynamics of olfactory dysfunction when the vomeronasal (VNE) and main olfactory epithelium (MOE) are compared. Methods. We used male rats of transgenic line 22 (TG22) of the bacterial artificial chromosome Huntington disease model (BACHD), aged 3 days or 6 months. Cell proliferation, apoptosis and macrophage activity were examined with immunohistochemistry in the VNE and MOE. Results. No differences were observed in cellular parameters in the VNE between the groups. However, the MOE of the 6-month-old HD animals showed a significantly increased number of mature olfactory receptor neurons. Other cellular parameters were not affected. Conclusions. The results obtained in the TG22 line suggest a relative stability in the VNE, whereas the MOE seems at least temporarily affected.

## TUNICAMYCIN SUPPORT THE CISPLATIN-INDUCED APOPTOSIS OF UVEAL MELANOMA CELLS

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Ocular melanoma is an extremely aggressive and rapidly metastatic uveal neoplasm. Unlike skin melanoma, its development is accompanied by a small number of genetic changes. This results in a poor response to immunotherapy, which is relatively effective in skin melanoma. The best chances for remission are rapid surgical intervention involving enucleation but the mortality rate still reminds at 45%, because of late metastases, appearing mostly to the liver. In our research we tested the possibility of inducing apoptosis in uveal melanoma cells using combinations of tunicamycin and cisplatin. Tunicamycin, a selective *N*-glycosylation inhibitor that causes endoplasmic reticulum stress, was already successfully used in combination with cisplatin to induce apoptosis in lung cancer cells. In our study uveal melanoma cell lines: 92.1 and Mel202 and cutaneous melanoma cell lines: IGR-39 and FM-55P were treated with tunicamycin (0.625 or 15  $\mu\text{g/ml}$ ) and/or cisplatin (25 mM) for 24h. Cell apoptosis was evaluated with the use of Annexin V-PI and Caspase3/7 activity assays. Both tests confirmed stronger initiation of apoptosis in melanoma cells by synergic stimulation with tunicamycin and cisplatin than using tunicamycin and cisplatin separately. However, cutaneous melanoma cells responded more to synergistic tunicamycin/cisplatin treatment in Annexin V/PI assay than uveal melanoma cells. But the latter cells showed more abundant caspase-3 activation. These results are promising in terms of usability of tunicamycin in uveal melanoma therapy scheme. However, the specific mechanisms of induction of apoptosis initiated by such synergistic treatment needs to be elucidated.

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## VARIED ASPECTS OF STEREOTYPE MANIFESTATION IN CAGED ANIMALS

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Animals which inhabit cages suffer from a behavioral disorder called stereotypy. The development of stereotypes occurs in the central nervous system. Its main part is the neural pathway between the striated body and the black substance. Other parties involved in stereotypy creation are structures of the central nervous system such as corpus amygdaloideum. The key factor of stereotypy occurrence is related to disorders of the neurotransmitters dopamine, serotonin and opioids in many areas of the brain.

Stereotypy is defined as unchanging, repetitive behavior and behavior that is used in productive life and functions of animals. Stereotypies could result in animal's self-mutilation, and motor agitation. The conception of stereotypy formation and its prevention is worth investigating as it affects the well-being of animals of any kind.

The aim of the study was to investigate the process of stereotypies formation. As a test subject, *Mandrillus sphinx* family in the Silesian Zoological Garden in Chorzów (SZG) was chosen. Observations were conducted for six consecutive days during tourist season. During these, strong stereotypical behaviour, made by one of the females who was excluded from the family, was observed. When noise had raised in the Chorzów SZG, female clung her limbs to the wall and stayed in this position for hours. The female also pulled her hair out in a self-harming manner. Other individuals of the family also manifested self-mutilation when noise level rose.

The obtained results suggest that occurrence of stereotypes is strictly related to noise and other disturbances of the animals milieu.

## **OROTIC ACID REDUCES VIABILITY BY ACTIVATING APOPTOSIS IN HUMAN OVARIAN GRANULOSA TUMOR CELLS**

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Orotic acid is a natural product and is known to be involved in many biological processes. Sheep milk is a rich source of orotic acid, which acts as a precursor in the pyrimidine nucleotide biosynthesis pathway. Most articles concerning the administration of orotic acid and its salts are focused on therapeutic effects, but their effect on tumors is not clearly defined. Some experimental data support the view that orotic acid has tumor-promoting properties, at least for experimental liver carcinogenesis. But in mice, orotic acid was shown to reduce the carcinogenetic effects of methylcholanthrene, which can cause ovarian cancer.

There are no data available on reproductive and developmental toxicity. Therefore, we elucidated the effects of orotic acid on the human adult granulosa cell tumor-derived KGN cell line. Cells were cultured in two-dimensional monolayer culture and exposed to orotic acid in doses 10, 25, 50, 100 and 250 nM for 24, 48 and 72h. Viability and apoptosis level were measured with the alamarBlue Cell Viability assay and the Caspase-Glo® 3/7 assay. We showed that higher doses of orotic acid reduced cell viability after 24, 48 and 72 hours; parallel with increased caspase 3/7 activity.

In conclusion, orotic acid treatment induced cell death, and may therefore, be a future therapeutic strategy for the prevention and treatment of ovarian granulosa tumor.



# **ROLE OF *PORPHYROMONAS GINGIVALIS* AND ITS VIRULENCE FACTORS IN mRNA TURNOVER REGULATION IN GINGIVAL FIBROBLASTS**

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Periodontitis involves chronic inflammation, which is caused by microbiota shift. In consequence, it may lead to gingival and bone tissue degradation. The anaerobic bacterium *Porphyromonas gingivalis* (PG) is a keystone pathogen in the development of periodontitis.

Changes in mRNA stability are one of the key mechanisms that regulate gene expression during inflammation. The process of regulating mRNA degradation may be, among other factors, mediated by RNA binding proteins. In 3'UTR regions of mRNA, there are regions rich in adenine and uridine - AU-rich elements (ARE). ARE are cis-elements that interact with trans-elements -ARE binding proteins (AUBPs). These proteins can stabilise or destabilise transcripts. Among AUBPs, tristetraprolin (TTP) family proteins and K-homology splicing regulator protein (KSRP) mainly destabilise mRNAs of pro-inflammatory mediators. Different isoforms of AU-binding factor1 (AUF-1) can either stabilize or destabilize mRNAs, whereas Hu antigen R (HuR) mainly stabilizes transcripts.

This study aimed to investigate both the kinetics of the expression rate and changes in mRNA stability of inflammatory mediators involved in periodontitis pathogenesis: interleukin-6 (IL-6), IL-8, and cyclooxygenase-2 (COX-2), C-X-C motif chemokine ligand 2 (CXCL2) in gingival fibroblasts (GFs). Moreover, changes in the expression AUBPs were analyzed. TTP expression appeared to be regulated by IL-1 $\beta$ , PG and fimbriae protein A (FimA) on mRNA and protein levels.

Obtained results suggest that mRNA stabilization plays an important role in GFs activation by cytokines, but the involvement of changes in transcript stability upon infection with PG appears to be more complex and requires detailed study.

## **SUPPLEMENTATION WITH POLYUNSATURATED FATTY ACIDS (PUFA) AFFECTS LEUKOCYTE TELOMERE LENGTH IN THREE MONTHS OLD MICE**

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Telomeres are repetitive ribonucleoprotein complexes present at the ends of chromosomes. They are considered a biological clock, as they shorten in parallel with aging. Challenging remains: how can we prevent accelerated telomere attrition and subsequent premature replicative senescence? Recent studies suggest a role of polyunsaturated fatty acids (PUFA) in this process. Therefore, we tested how dietary intervention with PUFA affect the leukocyte telomere length, indicator of biological ageing. For this purpose, we used male Swiss-Webster mice (n = 20). Mice until the third month of life were fed fodder differing in fatty acids (FA) content. Animals of the control group were fed with standard diet while another group was supplemented with oil additives. The FA composition of the experimental diet was as follows: saturated fatty acids (SFA): 9.91%; monounsaturated fatty acids (MUFA): 10.4% and PUFA: 79,69%. Diet was high in omega-3  $\alpha$ -linolenic acid (ALA) what resulted in low omega-6 linoleic acid (LA)/ALA ratio 5:1. The gas chromatography performed on liver, the central organ for FA metabolism, confirmed significantly higher level of omega-3 and omega-6 FA as well as lower LA/ALA ratio in mice supplemented with PUFA comparing to control group animals. Mice supplemented with PUFA demonstrated longer leukocyte telomeres than mice fed control diet. Obtained results confirm the role of PUFA in telomere biology and support a need for further studies on their use in the prevention of premature ageing and diseases related to telomere dysfunctions.

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# **NEW NON-SELECTIVE ROCK/AKT KINASES INHIBITORS – AN APPROACH TO DEVELOP ANTI-METASTATIC AND ANTI-INVASION DRUG CANDIDATES**

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Cancer cells are characterized by uncontrolled growth, rapid proliferation, and potential spreading to other parts of the organism, which is called metastases. The novel approach to cancer therapy assumes, that the cytostatic and antiproliferative treatment should be complemented by agents targeting motility, migration and/or invasion that are related to metastasis. As of today, no anti-metastatics have been introduced to cancer treatment. However, among agents targeting actin polymerization and function, two promising anti-metastatic drug candidates were found: CCT129254 and AT13148. Both compounds act by non-selective inhibition of Rho-associated protein kinases (ROCK1 and ROCK2) and protein kinase B (Akt1, Akt2 and Akt3). ROCK and Akt enzymes belong to two independent intracellular signalling pathways regulating actomyosin cytoskeleton contractility and their inhibition results in impairment of cells motility and mobility. Although targeting the same enzymes, CCT129254 and AT13148 differ significantly in potency and effects. CCT129254 more potently inhibits AKT2 in comparison with ROCK isoforms, while the AT13148 represents opposite profile of activity being a strong inhibitor of ROCK1 and ROCK2 and moderate inhibitor of three AKT isoforms. The aim of our study was to search for dual ROCK-AKT inhibitors with the balanced profile of activities to find new metastatics candidates. Based on CCT129254 structure, we designed and synthesized a series of 6 compounds. Based on *in vitro* biological studies among them we found a “hit” multi-kinase inhibitor, that will be a promising starting point for further studies.

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# HEME METABOLISM IN MATURING OOCYTES AND PREIMPLANTATION EMBRYO DEVELOPMENT

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Recently, it has become more apparent that cellular metabolism and its regulation may be a deciding factor governing proper preimplantation embryonic development and oocyte maturation. Changes in cellular metabolism are precisely timed and depend on the embryo development stage. Heme is crucial for healthy mitochondria, but its synthesis consumes glycine and succinyl-CoA. Although levels of labile heme in oocytes and embryos may play an essential role in their biology, the role of heme in developmental biology has been largely neglected so far.

Our data show that  $\delta$ -aminolevulinic acid ( $\delta$ -ALA), a heme precursor, stimulates the maturation of mouse oocytes but inhibits preimplantation embryonic development. Mouse embryos cultured with 350  $\mu\text{mol/L}$   $\delta$ -ALA are photosensitized and show inhibited cleavages when kept in the dark. The effects of  $\delta$ -ALA on embryo development are reversed with succinylacetone, which inhibits  $\delta$ -aminolevulinic acid dehydrogenase.

Preimplantation embryos can take up hemin and N-methylmesoporphyrin and up-regulate *Hmox1* in response to hemin or  $\delta$ -ALA. Finally, stimulation with heme accelerates embryonic development.

On the basis of our data and available literature, we hypothesize that labile heme is a significant regulator of oocyte and embryonic metabolism and the embryonic cell cycle.

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## **DOES PRENATAL IRON DEFICIENCY AFFECT THE COURSE OF NEONATAL HAEMOLYSIS IN MICE?**

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Mild intravascular haemolysis (in humans, called also neonatal jaundice) affecting all pre-term and a high proportion of term newborns, is a feature of normal newborn physiology. Neonates born with a certain amount of foetal erythrocytes which are rapidly replaced by adult erythrocytes. Impermanence of foetal red blood cells is caused by high instability of foetal haemoglobin in oxidative environment. The disruption of foetal erythrocytes is associated with the release to the blood-stream of highly reactive pro-oxidants, such as free haemoglobin (Hb) and its porphyrin component, heme. Free haem is neutralized by haem oxygenase-1, enzyme which cleaves the porphyrin ring, at the expense of molecular oxygen, to release ferrous iron, CO and biliverdin converted to bilirubin. In newborns, iron from foetal erythrocytes is recycled to the circulation, transported to bone marrow, and used in process of erythropoiesis. In the neonatal period supply of exogenous iron is inadequate due to functional immaturity of the molecular mechanisms of iron absorption. It is also well known that iron is the microelement indispensable for normal growth and development of young organisms, and during the pregnancy this microelement is transported from the maternal circulation to the foetus by placenta. However iron deficiency anaemia is a global health problem, which particularly affects pregnant women. Therefore in the present study using a mouse model, we investigated effect of iron deficiency in pregnancy on the course of intravascular haemolysis in the mouse neonates born from the anaemic mothers.

### ACKNOWLEDGEMENTS

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# **LINE-1 AMPLIFICATION IN LONG-RUN DNA-DAMAGE QUANTIFICATION (LORD-Q) ALLOWS ASSESSING INTEGRITY OF SPERM GENOME**

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DNA integrity is a very important parameter of sperm quality determining fertilization efficiency and proper development of the future embryo. This parameter is routinely assessed in medical diagnosis and research studies with a variety of tests which do not include, however, LORD-Q assay. LORD-Q (*long-run real-time PCR-based DNA-damage quantification*) is a very sensitive and fully quantitative method based on amplification of a short (40-70 bp) and a long (3000-4000 bp) fragments of the studied genome. A short fragment, which amplification is not inhibited by DNA damage, serves as normalization control. A long fragment, which amplification is inversely proportional to the amount of DNA lesions in DNA sample, serves as experimental probe. In the present study we tested whether LORD-Q is applicable for measurement of DNA damage in sperm. DNA isolated from mouse sperm was untreated (control) or subjected to various degrees of fragmentation. Then real-time PCRs were run using primers amplifying short and long sequence of LINE-1 repetitive elements. Amplification of LINE-1 was our innovative modification of the original method, where amplification of a single gene is applied. Since LINE-1 elements are spread in thousands of copies along genome, using them in the analysis increases accuracy and allows reducing the amount of input DNA template. The obtained results precisely reproduced the actual fragmentation of all analyzed samples, which proved that the modified method works properly and can be used to quantify DNA damage in sperm. LORD-Q should be considered as good alternative to traditional semi-quantitative tests of sperm genome integrity.

## MIRNA REGULATES THE ACTIVITY OF *PXT1* GENE

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Mouse *Pxt1* (*Peroxisomal, testis specific 1*) belongs to a group of proapoptotic genes encoding for a BH-3 like containing protein. It's expression is restricted to primary spermatocytes. Targeted deletion of this gene resulted in increased proportion of sperm with enhanced DNA strand breaks. Bioinformatic analysis revealed that miRNA particles may have putative binding capacity to *Pxt1* mRNA. Testis expression of all miRNAs candidates was confirmed *in vivo*. To assess, whether candidate miRNA particles have influence on *Pxt1* gene expression we used reporter assay based on luciferase activity. We have amplified 3'UTR part of *Pxt1* gene, which was then inserted into plasmid containing Firefly luciferase open reading frame and Renilla luciferase gene for normalisation. Using this construct we have transfected MC3T3-E1 mouse bone marrow cell line and demonstrated that 3'UTR of *Pxt1* gene interacts with miRNAs. Then, using a site directed mutagenesis mutations in several motifs predicted to bind miRNAs were introduced. The luciferase reporter assay showed that miRNA6996 regulates activity of *Pxt1*.

### ACKNOWLEDGEMENTS

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## **ANALYSIS OF MORPHOLOGICAL CHANGES IN CHO-K1 CELLS AS A RESULT OF FEXOFENADINE ACTION**

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Fexofenadine is a representative of a new generation of antihistamines, which, compared to their parent compounds, have several times greater H1 receptor blocking activity and are devoid of most side effects. The high therapeutic efficacy of these drugs, as well as the proven safety even with long-term use, have in recent years become the reason for expanding research on these compounds in terms to discover new mechanisms of action.

The conducted studies were aimed at demonstrating new mechanisms of action of fexofenadine at the cellular level. For this purpose, CHO-K1 line cells were incubated for 48 hours with fexofenadine in the concentration range of 7-300  $\mu\text{M}$ . The visualization of morphological changes in the tested cells was made by H&E staining using the Nikon Eclipse 80i optical microscope. Additionally, the mitotic index was determined and the MTT test was performed.

On the basis of the obtained results, it was found that fexofenadine induces concentration-dependent vacuolization and apoptotic changes in the tested cells, clearly promoting apoptosis at concentrations of 250 and 300  $\mu\text{M}$ . The cytotoxic effect of fexofenadine was confirmed by the reduction of the mitotic index and the metabolic activity of the tested cells, with the lowest values being found at 300  $\mu\text{M}$ . On the other hand, the presence of abnormal mitotic figures, binucleated and multinucleated cells, and directing damaged cells to the apoptotic pathway indicates a mitotic catastrophe. The induction of the above-mentioned cellular processes by fexofenadine may play an important role in extending the clinical application of this compound.



## **DANTHRON INDUCES MITOTIC CATASTROPHE IN CERVICAL CANCER CELLS**

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Neoplastic diseases are one of the causes of mortality, and factors that increase predisposition to its development include poor diet or stress. Hence, it is important to use products of plant origin, which often contain substances that induce various types of cell death, which may contribute to minimizing the risk of cancer. Such compounds may include anthraquinones, including the test danthron, which has multiple pharmacological properties.

The aim of the research was to assess the effects of danthron on HeLa cells and to try to demonstrate its potential anticancer properties.

The study was conducted on cervical cancer cells (HeLa line), which were treated with danthron in the concentration range of 50  $\mu\text{M}$  - 300  $\mu\text{M}$  for 48 hours. Morphological changes in cells were assessed using optical microscopy (H&E staining), which showed the presence of mitotic death markers. However, using fluorescence microscopy (DAPI staining), apoptotic changes were confirmed.

Morphological analysis showed a correlation between the anthraquinone concentration used and the increase in mitotic catastrophe, as evidenced by the increased number of bi- and multinucleated cells, as well as giant cells and micronuclei.

The presence of abnormal mitotic figures has been shown, including tripolar metaphase or multipolar anaphase, which indicates the effect of danthron on the cytoskeleton of the studied cells.

At the same time, it was found that at high concentrations (300  $\mu\text{M}$ ), danthron increased apoptosis, which was confirmed in fluorescence microscopy by the presence of cells with both chromatin condensation and cell nucleus fragmentation.

In summary, danthron induced mitotic catastrophe and apoptosis, processes relevant to anticancer therapy.

## C-REACTIVE PROTEIN IN GRAVES' DISEASE

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Graves' disease (GD) is becoming an increasingly important health problem. Besides elevated level of anti-TSHR antibodies and hyperthyroidism, GD is characterized by inflammatory response to tissue damage of the gland. One of the non-specific responses to disturbances in homeostasis is the acute-phase reaction involving C-reactive protein (CRP), which is mainly synthesized by the liver in response to IL-1 $\beta$  and IL-6. Physiologically, CRP is secreted into the blood in low concentrations and does not undergo glycosylation in contrast to inflammation when N-glycans were identified in CRP. Furthermore, CRP levels have been shown to increase in hyperthyroidism and to normalise during euthyroidism. Therefore, the aim of the study was to evaluate the CRP protein levels in the sera of GD donors before and after stabilisation of TSH levels as a result of methimazole treatment, as well as a preliminary analysis of CRP N-glycosylation.

Serum was obtained from patients before treatment (GD) and after TSH normalisation (GD/L), and healthy subjects (CTR). CRP was detected by immunoblotting, while the content and composition of CRP N-glycans were assessed by enzymatic de-N-glycosylation and lectin precipitation.

CRP was confirmed in the study groups and its lower content was observed in methimazole-treated donors. CRP was shown to be glycosylated in GD, with sugar structures enriched in sialic acid and fucose. Furthermore, immunosuppressive treatment was found to attenuate fucosylation and to increase the sialylation of CRP.

This study provides the preliminary data on the oligosaccharide structures of CRP and the changes in its glycosylation as a result of GD treatment.

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# THE EFFECT OF AGGREGATED $\alpha$ - SYNUCLEIN ON CYTOCHROME C RELEASE FROM MITOCHONDRIA: ROLE OF PROAPOPTOTIC PROTEINS BAX AND BAK

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$\alpha$ -Synuclein aggregation and mitochondrial dysfunction commonly appear in sporadic and inherited form of Parkinson's disease (PD), which is the second most common neurodegenerative disorder. The fibrils of  $\alpha$ -synuclein play a major role in this pathology by inducing the death of dopaminergic neurons but mechanism leading to neurodegeneration is still unclear. One hypothesis assumes that  $\alpha$ -synuclein fibrils may directly induce apoptosis, either by itself or by activating the pro-apoptotic proteins Bax and Bak. During the process of apoptosis one crucial point is cytochrome c release from mitochondrial intermembrane space (IMS) into cytosol.

Using *in vitro* model, I performed mitochondria isolation from MDA-MB-231 cells and incubation with  $\alpha$ -synuclein monomeric, oligomeric states and  $\alpha$ -synuclein PFFs (preformed fibrils) at concentrations of 0.5, 1 and 2  $\mu$ M, followed by centrifugation to estimate cytochrome c release from mitochondria by Western blot technique. The second part of my research was co-immunoprecipitation (co-IP) of Bax and  $\alpha$ -synuclein recombinant proteins. My results show that external application of pathological forms of  $\alpha$ -synuclein, but not monomers, promote release of the cytochrome c from isolated mitochondrial organelles from wild type (WT) cells and this effect appeared to be reduced in mitochondria from Bax/Bak double knock-out (DKO) cells, although the co-IP experiments don't prove interaction between  $\alpha$ -synuclein and Bax. It indicates that Bax and Bak proteins are necessary for  $\alpha$ -synuclein to induce cytochrome c release, thus leading to mitochondrial dysfunction, however the mechanism of this interaction remains unrevealed.

# **PROANGIOGENIC EFFECT OF MELANOMA-DERIVED ECTOSOMAL INTEGRINS DEMONSTRATED BY TUBE FORMATION ASSAY**

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**Background:** Integrins are known to play a crucial role in the initiation, progression, and metastasis of solid tumors. The aim of this study was to investigate whether the integrins delivered via melanoma-derived ectosomes have the ability to induce angiogenesis.

**Methods:** Ectosomes released by primary WM115 and metastatic WM266-4 cutaneous melanoma cells were isolated from conditioned media that had previously been concentrated by low-vacuum filtration by differential centrifugation. Then WM115- and WM266-4 derived ectosomes alone, or in combination with anti- $\alpha v \beta 3$  or anti- $\alpha v \beta 5$  integrin antibodies, as well as with cilengitide or echistatin, which are integrin antagonists, were added to endothelial cells (HUVEC) seeded on Matrigel. After 18 hours of incubation, images were taken and tube formation analysis was performed using Image J software with the addition of the angiogenesis plugin. Parameters such as the number of closed tubes, junctions, branching points, and total tube length were evaluated.

**Results:** The addition of melanoma-derived ectosomes increased the number of closed tubes, while the addition of ectosomes with integrin antagonists significantly reduced their number. WM115-derived ectosomes increased the overall length of the tubes, but the effect was diminished when anti- $\alpha v \beta 3$  or anti- $\alpha v \beta 5$  antibodies were added. Moreover, the addition of ectosomes in combination with anti-integrin antibodies or integrin antagonists generally resulted in a reduction in the number of junctions.

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# **INDUCTION OF APOPTOSIS AND CELL CYCLE ARREST IN ACUTE MYELOID LEUKEMIA CELLS BY GEMTUZUMAB OZOGAMICIN (MYLOTARG)**

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Gemtuzumab ozogamicin (Mylotarg, GO) is an antibody–drug conjugate (ADCs) composed of a cytotoxic agent, calicheamicin derivative, linked to a recombinant humanized antibody (IgG4) directed against the CD33 antigen. ADCs are a new class of anticancer agents commonly described as the “Trojan Horses” of therapeutic approach that deliver cytotoxic agents in a safe and efficient way to the target. In the case of acute myeloid leukemia (AML), the CD33 antigen is an appropriate target because it is expressed on AML blast cells in more than 90% of patients, whereas hematopoietic stem cells, lymphoid cells, and nonhematopoietic cells do not show its expression. Once bound to CD33 antigen, GO is rapidly internalized, followed by release of the calicheamicin derivative inside the lysosomes. The free calicheamicin binds to the DNA, causing double-strand DNA breaks. Herein, we have investigated and compared the effects of GO on four AML cell lines of different French-American-British types (KG-1, HL-60, ML-1 and MV4-11). The expression of CD33 antigen on leukemia cells was determined using immunocytochemical technique. AML cells were exposed to different concentrations of GO (10-10 000 ng/ml) and after 24h and/or 48h cell viability, cell cycle and apoptosis of leukemia cells were analysed using PrestoBlue assay, propidium iodide staining (PI) and annexin V/PI assay, respectively. Exposure of AML cells to GO caused dose-dependent decrease of cell viability. Moreover, this ADC induced G2 arrest and apoptosis of leukemia cells, however, the degree of cell response was variable and dependent on AML cell line used. Importantly, different cell responses did not correlate with the levels of expression of CD33 antigen. Thus, further studies are needed to evaluate the molecular pathways induced by the drug in AML cells.

## ACKNOWLEDGEMENTS

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# MATRIX METALLOPROTEINASE 16 IN THE CHICKEN OVARY

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The present study was undertaken to examine whether transcript levels of Matrix Metalloproteinase 16 (MMP-16) change during follicle development and whether gonadotropins and estrogen are involved in this enzyme regulation. In the first experiment (I) birds were sacrificed at two stages of the ovulatory cycle: 22h and 3h before ovulation of F1 follicle. In the second experiment (II), control hens received NaCl and experimental hens were injected daily with equine chorionic gonadotropin (eCG) at dose of 75 IU/0.2 mL of 0.9 % NaCl /kg b.w. In the third experiment (III), the control group was treated with a vehicle (ethanol) and the experimental group was treated daily with Tamoxifen (TMX) at a dose of 6 mg/kg of b.w. Chickens from experiments II and III were sacrificed on day 7 and 8, respectively. Expression of MMP-16 was examined by RT-PCR in white, yellowish, small yellow and in the theca and granulosa layers of the largest, preovulatory (F3-F1) follicles of control and treated groups. The relative mRNA expression (RQ) of MMP-16 was depended on follicular size and the layer of follicular wall. The higher expression of MMP-16 mRNA in the granulosa layer 3h compared to 22h before ovulation of F1 follicle was found. Administration of eCG decreased transcript abundance of MMP-16 in white and small yellow follicles, as well as in the theca layer of F3-F2 and the granulosa layer of F1 follicle. TMX caused an increase in mRNA expression of MMP-16 in the theca layer of F3-F1 follicles and a decrease in the granulosa layer of F1 follicle. The findings suggest that MMP-16 might participate in the ECM remodeling during follicle development and may be engaged in F1 follicle rupture during ovulation. Moreover, gonadotropins and estrogen may have a role in the regulation of the transcription of MMP-16.

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7. Brief communications are short papers (1–4 printed pages) reporting new findings that do not need a standard full-length treatment with the usual main headings. Brief communications are subject to normal review.
8. **References** in the text should be cited in the following form: Newton (1990) or Newton and Berrie (1982) or (Ward, 1950; Hiroshi and Ohta, 1970). For three or more authors, use the form Zinkowski et al. (1991) or (Zinkowski et al., 1991).

**Examples of style for references:**

**a) citations of journal papers:**

PALMER TP. 1962. Population structure, breeding system, interspecific hybridization and allopolyploidy. *Heredity* 17: 278-283.

CHEN BY, HENEEN WK, SIMONSEN V. 1989. Comparative and genetic studies of isozymes in resynthesized and cultivated *Brassica napus* L., *Brassica campestris* L., and *B. alboglabra* Baitey. *Theoretical and Applied Genetics* 77: 673-679.

**b) citations of books, congress proceedings, theses:**

BERGGREN DJ. 1981. *Atlas of Seeds*, part 3. Swedish Museum of Natural History, Stockholm.

BING D, DOWNEY RK, RAKOW GFW. 1991. Potential of gene transfer among oilseed *Brassica* and their weedy relatives. *Proceedings of the GCTRC Eighth International Rapeseed Congress*, 9-11 July 1991, 1022-1027. Saskatoon, Saskatchewan.

ROMEO JT. 1973. A chemotaxonomic study of the genus *Erythrina* (Leguminosae). Ph.D. dissertation, University of Texas, Austin, TX.

**c) citations of articles and chapters from books:**

PHILLIPS RL. 1981. Pollen and pollen tubes. In: Clark G [ed.], *Staining Procedures*, 61-366. Williams and Wilkins, Baltimore, MD.

Authors' names in References should be written in small caps.

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