

## Abstracts

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### Tolerability of inhaled N-chlorotaurine – a phase I clinical study

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N-chlorotaurine (NCT), an endogenous antiseptic applicable to different body regions was tested on its tolerability upon inhalation in humans. This was a double-blind and randomized study with a parallel test (1% NCT) and control group (0.9% NaCl as placebo) in two Austrian centers, the hospitals Natters and Vöcklabruck. Healthy, full age volunteers were included, 12 in each center. Exactly the half of each group was treated in each center. One inhalation (single dose 1.2 ml each, inhaled for 10 min) was done on every day on 5 consecutive days using an AKITA JET nebulizer.

Primary criterion of evaluation was the forced expiratory volume in the first second (FEV1). Secondary criteria were subjective sensations, further lung function parameters such as airway resistance, physical examination, and blood analyses (gases, electrolytes, organ function values, pharmacokinetic parameters taurine and methionine, immune parameters).

All included 15 females and 9 males completed the treatment and the control examinations according to the study protocol. FEV1 and all other objective parameters remained unchanged and constant during the treatment and in control examinations 1 week and 3 months after the treatment. Subjective mild sensations with a higher frequency in the test group were chlorine taste ( $P < 0.01$ ) and occasional tickle in the throat ( $P = 0.057$ ). Taurine, methionine, neopterin, tryptophan, and kynurenine plasma concentrations did not change within 60 min after inhalation or later on.

Inhaled NCT is well tolerated with only mild, topical and transitory subjective side effects.

### Biomarkers of immune and inflammatory response and the response to therapy with immune checkpoint inhibitors in patients with solid tumors

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The advent of immune checkpoint inhibitors like Ipilimumab or Nivolumab has virtually transformed the management of solid tumors. However, only a minority of patients does respond to this toxic and expensive therapy, and the search for reliable predictive biomarkers of immunotherapy reflects an unmet medical need. In the present study, serum C-reactive protein (CRP) and neopterin concentrations as well as peripheral blood cell count (PBC)-derived ratios were studied in melanoma patients treated with Ipilimumab. First preliminary data from this pilot in 28 patients with metastatic melanoma are presented. At baseline as well as during the treatment both urinary neopterin and CRP significantly correlated with PBC-derived ratios and lactate dehydrogenase (LDH). In multivariate analysis, neopterin, LDH and hemoglobin were significant predictors of survival. In conclusion, both CRP and serum neopterin correlate with PBC-derived ratios in this patient population treated with immunotherapy. Neopterin concentrations represent a significant independent predictor of outcome in melanoma patients treated with Ipilimumab.

## Effects of social stress on tryptophan-kynurenine metabolism in laying hens

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In modern housing conditions, birds kept for egg laying are separated from their mothers and relocated into large social groups consisting of thousands of individual birds, where establishing and maintaining social bonds

is difficult. Adverse social life events in mammals (social stress) such as maternal/peer-separation or social defeat, causes long term social incompetence and major disruptions in both immune and neurotransmitter systems, including the serotonin (5-HT) system. Feather pecking (FP), a widespread abnormal-repetitive behavior directed at the feather cover/skin of other birds, leads to integument injuries. It is suggested that FP is a tryptophan (TRP)/5-HT-related disorder. However, the TRP aetiopathophysiology is poorly understood. To help address this situation we investigated by HPLC and ELISA methods the relationship between psychosocial stress, activation of the TRP/kynurenine (KYN) pathway and FP behavior in an experimental line divergently selected for FP. We used 160 laying hens selected for high (HFP) or low (LFP) FP activity and an unselected control line (C). Birds were housed in floor pens in groups of 16 hens per pen (HFP; n=4, LFP; n=3, C; n=9, 10 pens). At 16 weeks of age, we disrupted the groups in 5 pens by mixing individuals with unfamiliar birds to simulate social stress. Blood plasma was collected one day prior to mixing and two days after mixing, to determine amino acid concentrations for TRP, phenylalanine to tyrosine ratios (PHE/TYR), KYN and the immune system biomarker neopterin. Aggressive and feather pecking were recorded, but not presented in the present paper. Data were analyzed using a GLIMMIX procedure in SAS. Social stress was associated with an 8% decrease in neopterin concentration (LSM 3.18 ± SE 0.05 vs LSM 2.92 ± SE 0.05, p < 0.05). Social stress did not affect TRP concentrations (LSM 105.14 ± 13.81 vs LSM 102.04 ± 26.71) but increased KYN levels by 17% (LSM 0.31 ± SE 0.02 vs LSM 0.37 ± SE 0.02, p < 0.05). PHE/TYR ratios were lower after social stress, however, only in HFP birds (0.75 ± 0.02 vs 0.70 ± 0.02, p < 0.05). KYN/TRP increased by 22% after mixing (LSM 2.99 ± SE 0.21 vs LSM 3.66 ± SE 0.21, p < 0.05). We therefore conclude that social stress may be associated with immune activation and changes TRP metabolism in stressed vs. unstressed birds, which may impact abnormal FP in birds kept for egg laying.

## Comparing plasma tryptophan measurements between labs using UPLC/HPLC approaches

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For a study that aimed at investigating the effects of social stress on plasma aromatic amino acid levels and tryptophan (TRP) metabolism in laying hens, we collected blood from 160 hens at two different time points ( $n_{\text{total}} = 320$ ). The amino acid analysis was performed in Toronto, SickKids Hospital (see [1] for detailed method description), on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Manchester, UK). The derivatized amino acids were detected at 254 nm. The Waters Acquity UPLC system employed consists of a binary solvent manager, a sample manager, a tunable, dual wavelength ultraviolet/visible (TUV) detector, and a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) C18 column ( $2.1 \times 100$  mm), column temperature was at 48°C. For the analysis of TRP and its metabolite kynurenine (KYN), aliquots of the same samples were shipped to Innsbruck, Austria and TRP and KYN concentrations were determined using a Model 9010 HPLC pump (Varian, Palo Alto, CA), controlled by a DS 654 data system. Reversed-phase cartridges LiChroCART RP<sub>18</sub> columns (244 mm length, 5  $\mu\text{m}$  grain size) from Merck (Darmstadt, Germany) were used. Tryptophan was detected by a fluorescence detector (Hewlett Packard, Model 1046A, excitation wavelength: 285 nm, emission wavelength: 365 nm); [2] for detailed method description, with modifications as specified in [3]. The TRP datasets gained from these two different analysis by two different labs (referred to as TRP\_T for Toronto and TRP\_I Innsbruck, [ $\mu\text{mol/L}$ ]) compare as follows: TRP\_T mean =  $109 \pm 15.9$  SD, Min = 49.2, Max = 151; TRP\_I mean =  $103 \pm 11.8$  SD, Min = 60.9, Max = 154. A PROC CORR procedure in SAS 9.4 revealed a strong positive correlation between the two methods ( $R=0.6$ ). In conclusion, while there appear differences between the datasets when looking at descriptive statistics, the two sets still show a significant correlation in spite of the different methods used to obtain the data, which seems especially remarkable since one method employed UV absorption detection of derivatized compounds, the other relied on direct (native) fluorescence detection.

[1] Birkl P, et al. *Poultry Sci* 2017;96:3021–5

[2] Widner B, et al. *Clin Chem*. 1997;43:2424–6

[3] Laich A, et al. *Clin Chem* 2002;48:579–81

## Pivotal role for endothelial cell GTP cyclohydrolase and tetrahydrobiopterin in cardiovascular adaptations to pregnancy

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Pre-eclampsia (PE) is characterized by endothelial dysfunction and abnormal uteroplacental remodelling and hypoperfusion. In pre-eclampsia, vascular nitric oxide (NO) bioavailability is reduced and markers of oxidative stress are increased. Tetrahydrobiopterin (BH4) is an essential cofactor for endothelial nitric oxide synthase (eNOS) activity; when BH4 availability is limiting, eNOS becomes uncoupled and generates superoxide instead of NO. We investigated the requirement for endothelial cell BH4-dependent eNOS regulation in the uterine vascular adaptation and blood pressure responses to pregnancy, using a mouse model with endothelial cell-specific deletion of *Gch1*, encoding GTP cyclohydrolase 1, an essential enzyme for BH4 biosynthesis.

Mice with a floxed *Gch1* allele (*Gch1*<sup>fl/fl</sup>) were crossed with Tie2cre mice to delete *Gch1* in endothelial cells, generating *Gch1*<sup>fl/fl</sup>Tie2cre mice. We demonstrated that vascular adaptation to normal pregnancy were associated with increased eNOS-derived NO-mediated vasodilatation in uterine arteries from pregnant wild-type mice. Deficiency in endothelial cell BH4 biosynthesis during pregnancy led to a loss of eNOS-derived NO mediated vasodilatation, and medial hypertrophy in uterine arteries from *Gch1*<sup>fl/fl</sup>Tie2cre mice. Deficiency in endothelial cell BH4 led to significantly increased blood pressure during pregnancy, and was associated with reduced placental size and with fetal growth restriction in litters born to *Gch1*<sup>fl/fl</sup>Tie2cre mice, independent of fetal genotype.

Taken together, these findings suggest a novel requirement for endothelial cell *Gch1* and BH4 biosynthesis in vascular adaptations and blood pressure regulation to pregnancy, and to placental function and fetal growth. Thus increasing endothelial cell BH4 biosynthesis may represent a novel therapeutic route to prevent and treat preeclampsia and/or improve placental function.

## Diverging gender differences in the associations between tooth loss, obesity and depression in the Old Order Amish

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Despite major preventative and treatment efforts, U.S. suicide rates are increasing, as does the prevalence of obesity. Suicide and obesity, through cardiovascular illness, are major contributors of premature death. Inflammation is implicated in suicidal behavior, depression, obesity, and cardiovascular morbidity and mortality. Considering the modifiable reciprocal interactions between dental health and inflammation, and gender differences in inflammation, as a stepping stone for future larger projects we have investigated gender differences in linking tooth loss and false teeth (as negative outcomes for several dental conditions), depression and obesity. Smoking and socioeconomic status have been associated with obesity (negatively), periodontal disease (positively), depression, and suicidal behavior, and are thus important potential confounders to our inquiry. We thus conducted our study in the Old Order Amish, a socioeconomically more homogenous population with very low prevalence of smoking.

In 4,708 Old Order Amish (2706 {57.48%} women) participants in the “Amish Wellness Program” in Lancaster PA, we recorded self-reported tooth loss and more recently, false teeth, measured weight and height, calculated body mass index (BMI) and ascertained obesity status. We also collected mood questionnaires based on PHQ 9 and PHQ 2, and transformed from ranked to binary the hopelessness/dysphoria and anhedonia item scores (current, past and ever) defined overweight status as BMI  $\geq 25$ , and Obesity

as BMI  $\geq 30$ . Statistics included multivariable linear and logistic regressions.

There was a significant gender X tooth-loss interaction ( $p = 0.005$ ) in relationship to BMI. Stratification by gender and adjustment for age, led to uncovering significant associations between tooth-loss and metabolic factors (BMI, obesity or overweight) in women ( $p < 0.05$  adjusted for age- for all three variables) but not in men. The collection of depression symptoms ratings in the Amish only started after the inquiry about tooth-loss ended, being replaced by inquiry regarding false teeth. Thus we do not have any data on associations between tooth loss and depression.

In contrast to tooth-loss, the association between false-teeth and metabolic factors was significant in men (adjusted for age, BMI  $p = 0.01$ , overweight  $p = 0.04$ , obesity NS) but not in women. Similarly, associations between false teeth and certain depression symptoms were significant in men (especially for past/ either anhedonia or hopelessness/ dysphoria, but not other depression scores, adjusted for age  $p = 0.03$ ) but not in women. Finally, the associations between depressive symptoms and metabolic factors were significant only in women, (adjusted for age, most robust associations were between obesity and current depressive symptoms - hopelessness/dysphoria, anhedonia, current both  $p < 0.01$  and current either,  $p < 0.005$ , and ever either  $p = 0.04$ ; for associations between overweight and obesity -current anhedonia  $p = 0.03$  and current either  $p = 0.01$ , with no significant associations with other depression symptom scores; no significant association with BMI). Limitations include a cross-sectional design not permitting causal inferences, and not adjusting the level of significance for multiple comparisons.

In conclusion, we found significant association between metabolic factors and a) tooth loss and b) depression to occur only in women, and between false teeth and a) metabolic factors and b) depression symptoms to be significant only in men. Some of our results replicate in the Amish gender differences reported elsewhere (e.g. tooth loss and BMI association stronger in women, but other associations are unprecedented). The social homogeneity in the Amish and virtual absence of smoking in the adult Amish population reduces the possibility of spurious associations based on these two strong confounders. Prospective designs, testing mechanistic inferences (microbial, immune, hormonal, dietary), may further result in identifying gender-specific treatment targets that could improve dental, psychiatric and metabolic domains, through better understanding their reciprocal interactions.

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## Hepatic HFE and iron-driven modulation of Kupffer cell kinetics critically affect cholesterol homeostasis

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Iron metabolism plays a crucial role in chronic diseases including atherosclerosis. Specifically, iron overload has been linked to an increased risk for atherosclerosis development. However, the underlying mechanisms are poorly understood.

By combining data from GWAS screenings in >20,000 individuals of European ancestry, genome editing of murine and human cells, and functional validation studies in mice, we identify the iron metabolism as an important regulator of cholesterol homeostasis. Dietary iron overload of apoE<sup>-/-</sup> mice lacking the hemochromatosis gene Hfe reduced plasma LDL-C levels, thereby inhibiting atherosclerosis development. *In vitro* experiments demonstrated Hfe to inhibit LDL receptor expression in hepatocytes, and iron to drive cholesterol transfer from Kupffer cells to hepatocytes in an apoA-I dependent fashion, which appeared to play a pivotal role in hepatic removal of LDL-C.

Our results define the iron metabolome as conserved regulator of cholesterol metabolism and identify Hfe as well as Kupffer cells as promising therapeutic targets to treat cardiovascular disease in humans.

## Urinary neopterin:creatinine ratios are sensitive indicators of infection in preterm infants suspected of infection in the absence of a clear diagnostic parameter

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There is an increased risk of infections and subsequent sepsis in premature infants due to incomplete maturity of the immune system. Detecting the presence of an infection is difficult since typical signs such as fever are often absent whilst traditional markers such as white cell counts may be skewed and cultures late in arriving. Where clear diagnostic parameters or reliable clinical signs are lacking a sensitive and early indicator for infection is necessary in preventing the high mortality and serious morbidity associated with sepsis. Increased serum and urinary concentrations of neopterin are associated with diseases where the cellular immune response is increased. Premature infants at risk of infection have been shown to have sudden increases in neopterin at the initial clinical suggestion of the diagnosis with concentrations increasing with severity of infection (1). Since premature infants require minimal handling, using urine for neopterin analysis has the distinct advantage of being non-invasive and can be easily collected.

This study monitored 21 premature infants through their stages of non-infection, suspected infection and proven infection. The infection was suspected based upon clinical judgement, whilst the infection with sepsis was diagnosed through positive cultures. Urinary samples were collected over 8 weeks and neopterin concentrations were determined by high liquid performance chromatography. Urinary creatinine (CR) was also measured to eliminate physiological variations of urine densities with differing body mass. The ratio of neopterin to creatinine (NPT:CR) was then correlated retrospectively with the medical records to identify which stage the infant was in at the time of collection.

The results showed an increase of urinary NPT:CR concentration from non-infection to suspected infection. This increased significantly further from the suspected infection stage to the confirmed infection stage. The concentration of urinary NPT:CR is a sensitive indicator of

infection in premature infants. Results confirm and extend earlier data [2,3]. Moreover, where there is a suspected infection on clinical judgement alone, the increase in the urinary NPT:CR ratio is a valuable diagnostic parameter of infection.

[1] Jürges, J & Henderson C. *Clin Exp Immunol* 1996;105:551-5

[2] Plata-Nazar K & Jankowska A. *Pteridines* 2011;22:77–89

[3] Plata-Nazar K, et al. *Acta Biochim Pol* 2015;62:133-7

## Immune regulation via the AHR-CYP1 feedback loop

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Environmental compounds originating from pollution or protective environments like in traditional farms influence the human immune system by multiple mechanisms. The transcription factor aryl hydrocarbon receptor (AHR) has modulating functions on multiple cell types and binds small molecules. By its activity Cytochrome P4501 (CYP1) act downstream of the AHR and metabolizes small molecules. Unclear is whether CYP1 activity is relevant for the modulation of the immune system as it is observed for the farm environment. We studied [1] the interdependence of CYP1 and AHR in human primary immune cells by using specific inhibitors of the Ahr and the CYP enzymes. CYP1 inhibition increased the expression levels of the stem cell factor receptor (c-Kit) and interleukin (IL)-22 while it decreased IL-17. Interestingly flow cytometric analysis revealed that CYP1 inhibition selectively promoted CD4<sup>+</sup> T cells that co-express c-Kit and IL-22 simultaneously. The addition of an AHR antagonist prevented these effects. In addition to T cells, also other human immune cells expressed CYP and generate a cell-specific fingerprint. We therefore hypothesized that similar mechanisms are present in multiple immune cells. The results illustrate a feedback loop in human immune cells where CYP1 inhibition resulted in an altered AHR-dependent immune response.

[1] Effner R, et al. *Sci Rep* 2017 Mar 9;7:44005

## The effect of kynurenic acid on C1q-CD59 balance in high glucose-exposed SH-SY5Y neurons via nitric oxide synthesis

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Activation of complement system causes cell death directly, because of the membrane deposition of the cytolytic neuron membrane attack complex (NMAC). C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation. Contrarily, CD59 is a natural membrane-bound inhibitor of the cytolytic NMAC. This endogenous complement regulating protein may be inactivated by glycation in the presence of high concentrations of glucose. Human neuroblastoma cell lines can constitute an *in vitro* model to analyze complement biosynthesis by human neurons. In this study, following the high glucose exposure, the effect of N-Methyl-D-Aspartate (NMDA) receptor blockade on glucose transporter3 (GLUT3), C1q, CD59, oxidative stress, nitric oxide ([NO<sub>3</sub>+NO<sub>2</sub>]; NO<sub>x</sub>) levels and total mitochondrial activity/neuronal viability was investigated. SH-SY5Y human neuroblastoma cells were exposed to (150-250 mg/dL) high glucose considering International Diabetes Federation criteria. Following NMDA receptor antagonist, kynurenic acid (KynA), and neuronal nitric oxide synthase inhibitor supplementation to SH-SY5Y cell culture medium, total mitochondrial metabolic activity/cell viability percentage, NO<sub>x</sub>, GLUT3, C1q and CD59 levels were measured, and oxidative stress coefficients were calculated. The total mitochondrial metabolic activity/cell viability was determined by MTT assay. NO<sub>x</sub> levels were measured by using Griess Method. The oxidative stress intensity coefficient (Q) was calculated. C1q, CD59 and GLUT3 levels were determined by ELISA. The high glucose supplementation to the cell culture medium increased oxidative stress, C1q and NO<sub>x</sub> levels, while cell viability decreased inversely. The increase in C1q and NO<sub>x</sub> levels prior to the rising of oxidative stress suggested that C1q warned the onset of high glucose-dependent oxidative stress. In the high glucose exposed SH-SY5Y cells, L-NAME caused the decrease in C1q and NO<sub>x</sub> synthesis,

whilst total mitochondrial metabolic activity/neuronal viability increased. The C1q and NOx levels were found to increase 2.5-fold compared to controls by the addition of glucose to the medium, whereas CD59 levels increased by 2.1-fold in comparison to controls. Contrarily, 30nM KynA addition to the high glucose containing medium provided 2.5 times decrease in C1q and NOx synthesis compared to the cells exposed to high glucose alone. In this case, CD59 decreased by 2.2-fold. C1q exerts a detrimental effect on neuronal viability, most likely through the activation of the classical complement cascade through the glucose toxicity. Additionally, it is thought that reactive nitrogen species generation by the glucose-induced NMDA receptor activation triggered C1q synthesis, prior to the CD59 changes in SH-SY5Y cells, however neurons express only low levels of CD59. During the high glucose exposure, oxidative stress and total mitochondrial metabolic activity/neuronal viability was tightly controlled by KynA through the NMDA receptor blockade.

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## Immunomodulatory properties of zinc oxide

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Zinc oxide (ZnO) nanomaterials are used not only in technical and chemical industry, but also pharmaceutical, food and cosmetic applications are frequent, due to the relatively low toxicity and biodegradability. While the use of ZnO and nanomaterials is considered not to pose a risk of adverse effects after dermal exposure, there are concerns regarding ZnO containing aerosols.

Human peripheral blood mononuclear cells (PBMC) as well as myelomonocytic cell lines such as THP-1 have been applied successfully to investigate immunomodulatory effects of compounds and materials. For such studies, the interferon-gamma-induced metabolic pathways of tryptophan breakdown to kynurenine via indoleamine 2,3-dioxygenase (IDO-1), and neopterin formation via GTP-cyclohydrolase can be used as readout. ZnO bulk material and nanoparticles were analysed in this *in vitro* setting in

a concentration range from 2.3 to 375 µg/ml. Nanoparticles and bulk materials showed different toxicity, the nanoparticles reducing cell viability in a concentration-dependent manner up to 70% with the highest concentration, at which the first effects of the bulk material became obvious. The kynurenine to tryptophan ratio, a measure of IDO-1 activity, decreased with all materials dose-dependently in mitogen-stimulated cells. However, nanomaterial treatment of unstimulated cells increased IDO-1 activity, while this effect was less prominent with the bulk treatment. Neopterin concentrations showed the same trend.

Data indicate that within a certain concentration range, ZnO nanoparticles may have some activating effect on unstimulated PBMC, while in stimulated cells and with higher concentrations immunosuppressive effects prevail. A closer elucidation of these effects is clearly warranted.

## A novel kynurenine-dependent circuit in DC1 promotes IDO1 expression in DC2 leading to suppression of experimental autoimmune encephalomyelitis

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DCs are potent T cell activators but they are also involved in maintaining immune homeostasis and self-tolerance and can be classified into three major types: pDC, DC1 and DC2. One mechanism by which DCs regulate tolerance involves indoleamine 2,3-dioxygenase 1 (IDO1) a tryptophan (Trp) metabolizing enzyme. In this study, we analyzed the ability of L-Kyn to induce tolerogenic IDO1 pathway in different DCs subsets *in vitro* and *in vivo* model of experimental autoimmune encephalomyelitis (EAE). We show that inflammatory *stimuli*, like LPS, were able to induce IDO1 only in DC1, but not in DC2 or pDC, when DCs were treated as isolated cultures. In contrast, when LPS was added to cultures containing all three

DC subsets, LPS could also induce IDO1 expression in DC2, which acquired tolerogenic function. Kynurenine produced by DC1 activates AhR in DC2 inducing IDO1 in a RelB-dependent manner. *In vitro* L-Kyn treatment impaired DC2 T cells priming ability causing suppression of MOG-specific reactivity with an increment of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells. *In vivo*, oral administration of L-Kyn induces functional T<sub>reg</sub> cells that suppress EAE and this effect is completely abrogated in Ahr<sup>flox/flox</sup> CD11C Cre<sup>+</sup> mice. These results suggest that in specific microenvironments, small numbers of IDO1-expressing DC1 may spread tolerogenic activity to DC2 cells through a kynurenine-AhR axis and L-Kyn could constitute a unique endogenous molecule for therapeutic immunomodulation of inflammatory and autoimmune diseases.

## Tryptophan, kynurenine and neopterin serum levels during dengue virus infection

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Dengue virus (DENV) disease is one of the most important arboviral diseases. DENV infection induces immune system activation including the production of the immune system biomarker neopterin [1] and the activation of the tryptophan (Trp) degrading enzyme indoleamine 2,3 dioxygenase-1 (IDO-1) [2,3], as is indicated by an increased kynurenine to tryptophan ratio (Kyn/Trp) in the blood of infected patients. In this study, serum samples from seventy-four patients with indication of DENV infection were screened for serum neopterin, tryptophan and kynurenine levels. Compared to healthy blood donors, the mean neopterin concentration ( $33.8 \pm 13.3$  nmol/L) was significantly elevated. Likewise, the mean Kyn/Trp ( $93.4 \pm 57.4$   $\mu$ mol/mmol) was significantly increased. Fifty-five patients were confirmed seropositive for Dengue virus, this group presented with significantly higher neopterin levels ( $36.5 \pm 11.7$  nmol/L) and Kyn/Trp ( $102.0 \pm 61.3$   $\mu$ mol/mmol) compared to individuals with seronegative testing results. The association between neopterin and Kyn/Trp concentrations was strongly positive and interestingly

it was even stronger in the seronegative (rl = 0.886,  $p < 0.001$ ) compared to the seropositive patients (rl = 0.366,  $p < 0.01$ ). Our study confirms and extends earlier findings about an important role of neopterin and tryptophan metabolism in the course of DENV infection. Of note, both biochemical pathways are strongly inducible by the Th1-type cytokine interferon- $\gamma$ . The strong correlation between neopterin and Kyn/Trp concentrations observed in the seronegative group of patients may indicate that they suffered from infections other than DENV, probably viral, which have not been screened for but evoked the immune response which underlies the parallel increase of neopterin and Kyn/Trp concentrations. The abnormal tryptophan metabolism in DENV patients could also influence serotonin availability in platelets and play a role in the pathogenesis of hemorrhagic fever. Future follow up studies will show whether the monitoring of neopterin and Kyn/Trp concentrations is able to reflect the course of infections in the patients and to predict outcome. Moreover, especially the changes of tryptophan metabolism are likely to influence serotonin production and may relate to the neuropsychiatric symptomatology in the patients with DENV infection.

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## Human vs. murine immune system – similarities and differences

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It is some 15 years ago that the mouse genome was sequenced, following on from the landmark sequencing of the human genome. Subsequent analysis has revealed that more than 90% of the mouse genome can be aligned with a region of the human genome, and that some protein encoding-regions show striking similarities (60 to 99% identity) [1]. The genomic information and the technological advances in mouse genetics further promoted the wide-spread use of mouse models to mimic

human diseases [2]. Some years later, the scientific discussion is focused upon the extent to which genomic responses in mouse models are able to mimic human inflammatory diseases [3,4], and about how to control the confounding effects of biased statistical analysis [2]. Thus, it is of great importance to be aware about functional similarities and differences between species, in particular when investigating immunological cascades.

Initially, the immune system relied on multi-purpose myelo-monocytic cells that were able to phagocytose and to present antigens, while the development of specific lymphocytes exerting innate and adaptive responses is an innovation of vertebrates [5]. Human, mouse and other mammals shared a common ancestor approximately 80 million years ago. Despite the genetic similarity and conservation of core biological processes, some substantial biochemical differences developed which lead to poorly correlated physiological responses.

One such example is the synthesis of the human immune activation marker neopterin [6]. Neopterin is produced by human and primate monocytes/macrophages via the enzyme GTP-cyclohydrolase I which is itself induced by cytokines such as interferon  $\gamma$ . Neopterin concentrations in human serum, cerebrospinal fluid and urine were found to be of prognostic and predictive value for a variety of diseases associated with immune activation such as infection, tumors, cardiovascular disorders and allograft rejection.

However, in other cell types such as fibroblasts, endothelial cells, as well as in rodent macrophages, the molecule tetrahydrobiopterin ( $BH_4$ ) is produced via the same biochemical pathway, as these cell types retain 6-pyruvoyl tetrahydropterin synthase activity, which is lost in human/primate monocytes/macrophages.  $BH_4$  is an essential cofactor for several monooxygenases of importance during immune activation, including nitric oxide (NO) synthase - NO suggesting peroxynitrite formation is substantially different in rodents compared with primates. Moreover, although a widely used biomarker in the human system, neopterin concentrations cannot be used to monitor disease in rodents. One humanized mouse model approach has been to transfer human peripheral blood mononuclear cells into severe combined immunodeficiency (SCID) mice [7], but also for such models the sources of neopterin and its value as a biomarker of disease has to be critically evaluated.

To summarize, similarities in the gene sets between two species do not automatically implicate transcriptional and functional similarities, due to regulatory interferences at different levels, notably at the metabolic level. Understanding important differences in the activation

of immunoregulatory cascades is of utmost importance to further decipher the fine-tuned regulatory network in human immunity; not only to avoid catastrophic and unforeseen biological actions of prototypic drugs in humans as it was the case with the immunomodulatory drug Theralizumab (TGN1412), but also, to once more broaden the use of alternative model systems.

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## Isolation and analysis of novel sulfated coumarins in the siphonous green alga *Dasycladus vermicularis* (Scopoli) Krasser

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The siphonous green algae form a morphologically diverse group of marine macroalgae which include two sister orders (Bryopsidales and Dasycladales) sharing a unique feature compared to other green algae as they are able to form large, differentiated thalli comprised of a single, giant tubular cell. Upon rupture a cascade of protective mechanisms have evolved including the extrusion of sulfated metabolites which are involved in the formation of a rapid wound plug [1]. We analyzed the composition of sulfated metabolites in *Dasycladus vermicularis*, Dasycladales which resulted in the isolation of four coumarins including two novel structures and 2 phenolic acids. In addition, an analytical HPLC assay for the quantification of those compounds was developed and performed on a Gemini C18 column. The analysis of several samples of *Dasycladus vermicularis* from different collection sites, water depths and seasons, revealed differences in the coumarin content ranging between 0.26 % to 1.61 % per g dry weight. In addition, the wound-healing activities of the major compounds from *D. vermicularis*

were investigated in a previously validated collagenase assay [2], indicating a dose dependent inhibition of the enzyme with  $IC_{50}$  values around 16.98  $\mu\text{g/ml}$ .

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## Antioxidative activity of the tryptophan metabolite hydroxyanthranilic acid

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Hydroxyanthranilic acid (HAA) is a metabolite of the tryptophan breakdown pathway along the kynurenine axis, and has prominent biological activities. The formation of this antioxidant during inflammation-induced tryptophan metabolism is suggested to be a protective mechanism against reactive oxygen species (ROS) that are generated in the course of the immune response [1]. HAA was shown to attenuate T helper (Th) type 1 cell proliferation and function [2]. As a barrier organ, skin is exposed to ROS damage frequently, and it has been shown that tryptophan metabolites are also present in the epidermis [3].

In this study we investigated the effect of HAA in human peripheral mononuclear cells (PBMC) and keratinocytes. Using PBMC as model it was shown that HAA could suppress mitogen-induced tryptophan breakdown at a concentration of 100  $\mu\text{M}$ , while unstimulated cells were not affected. This decrease was paralleled by a reduction of viability. The capacity of HAA to counteract ROS stress was assessed in HaCaT, a spontaneously immortalized human keratinocyte cell line. HAA decreased peroxy-radical induced oxidative stress in keratinocytes significantly and in a dose-dependent manner, with a half maximal inhibitory concentration ( $IC_{50}$ ) of approximately 80  $\mu\text{M}$ . Moreover, an attenuation of physiological ROS levels was observed, with an about 10 times higher  $IC_{50}$ . The cell viability was not affected in the tested concentration range (50 to 400  $\mu\text{M}$ ).

Our data confirmed the antioxidative activity of HAA in different cell models. HAA concentrations used in these experiments were relatively high compared to reported

serum concentrations. However, at sites of inflammation higher local concentrations may be reached. Further studies are necessary to elucidate the relevance of HAA as an endogenous antioxidant to avert radical-induced tissue damage in skin inflammation.

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## Probing the roles of orphan chemokines in macrophage function

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CXCL4 and CCL18 are two chemokines which have a pro-survival activity on monocytes, mediated by unidentified receptors. Both chemokines have been detected in atherosclerotic plaques, suggesting that they might contribute to the associated pathology. CXCL4 has been reported to induce monocyte polarization to a so-called "M4" phenotype which secretes CCL18 and is distinct from classically activated M1 macrophages and alternatively activated M2 macrophages [1]. We set out to further characterise the effects of these two chemokines on monocyte function.

Both CXCL4 and CCL18 significantly enhanced the survival of monocytes in serum-free media. In contrast to a previous report, CXCL4 treatment did not induce the expression of CCL18 by monocytes at mRNA or protein level, but was associated with a reduced ability to uptake oxidised low-density lipoprotein (oxLDL). CXCL4 treatment also induced expression of the chemokine CCL22, a recruiter of Th2 and Tregs. RT-PCR array analysis of transcription factors induced during the early stages of M4 differentiation points towards the activation of non-canonical NF $\kappa$ B signalling pathways. Collectively, our data suggest that M4 macrophages may a regulatory role in the atherosclerotic plaque, dampening inflammation and promoting remodelling, in contrast to a previously assumed atherogenic role.

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## Cell cycle regulation by CDK inhibitor proteins

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Signal transduction processes aiming to control cell proliferation need to connect to the machinery that coordinates and restricts cell cycle progression. At the centre of this machinery are protein kinases of the family of cyclin-dependent kinases (CDKs). Their activity is controlled by multiple mechanisms including the binding of CDK-inhibitory proteins. The CDK inhibitor p27<sup>Kip1</sup> binds and regulates CDK kinase activity in response to various mitogenic and antimitogenic signals. We recently observed a direct link between the erythropoietin/ erythropoietin receptor initiated signalling and p27, which provides a novel mechanism how erythropoietin stimulates cell proliferation.

The hormone erythropoietin (Epo) triggers erythropoiesis and activates intercellular signal transduction pathways that regulate proliferation, but also differentiation and survival of erythroid progenitor cells. Epo binds and activates the type I erythropoietin receptor (EpoR). We recently observed that p27 can directly bind to the EpoR *in vitro* and that endogenous p27<sup>Kip1</sup> is in a stable complex with EpoR in proliferating Epo-dependent erythroleukemic UT-7 cells. Upon Epo stimulation, p27 becomes rapidly phosphorylated on tyrosine 88. This leads to inactivation of the CDK-inhibitory activity of p27. This mechanism can contribute to the Epo-induced proliferation of erythroid progenitor cells. In addition, phosphorylation of p27 on this residue can also promote the ubiquitin-dependent proteasomal degradation of the CDK inhibitor protein.

Interestingly, the CDK inhibitor protein is also cleaved in cells undergoing programmed cell death in response to DNA damage. We observed that activated caspases cause proteolytic processing of p27. Caspase cleavage removes a C-terminal fragment of 22 amino acids from p27. This element harbours a phosphodegron that usually leads to cell cycle phase-dependent degradation of the CDK inhibitor. Removal of this fragment protects the inhibitor from SCF-Skp2 mediated degradation in the S, G2 and M phase of the cell cycle. The truncated protein becomes stabilized and remains an efficient nuclear inhibitor of cell cycle progression.

In addition to controlling cyclin/CDK kinase activity, p27 also regulates cytoskeletal dynamics, cell motility and cell invasion. Following processing by caspases, p27 fails to bind to RhoA and to inhibit its activation, and thereby abolishes the ability of p27 to stimulate cell migration and invasion. Caspases exert also non-lethal functions in diverse developmental processes including cell differentiation and tissue remodelling. The stabilization of the CDK inhibitor independent from the cell cycle position and the elimination of RhoA-induced cytoskeletal remodelling following caspase processing might also contribute to cell cycle exit and cytoskeletal remodelling during non-lethal caspase controlled differentiation processes.

## Low cerebral levels of tryptophan, phenylalanine and tyrosine are associated with depression in subarachnoid hemorrhage patients

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The availability and processing of aromatic amino acids tryptophan, phenylalanine and tyrosine seem to play an important role in the pathophysiology of depressive disorders. By using cerebral microdialysis (CMD), we measured brain extracellular levels of these amino acids in subarachnoid hemorrhage (SAH) patients and associated them with the presence of depression. Amino acid levels were analyzed daily using high performance liquid chromatography in 26 consecutive SAH patients with CMD. Patients were grouped according to the presence of depression: prior to the SAH, within 12 months after the SAH, or neither. Statistical analysis was performed using generalized estimating equations.

CMD-tryptophan (OR=0.68, CI95=0.55-0.83, p<0.001), CMD-phenylalanine (OR= 0.71, CI95=0.58-0.87, p=0.001)

and CMD-tyrosine (OR=0.7, CI95=0.56-0.88, p=0.002) levels were significantly lower in patients with preexisting depressive disorders compared to those without depression. CMD-tryptophan levels were moreover significantly lower in patients diagnosed with depression within 12 months after the SAH compared to those without depression (OR=0.78, CI95=0.63-0.96, p=0.02). Disease severity and SAH-related complications were not associated with amino acid concentrations. In patients without depression we found a positive correlation between nutritionally administered and brain interstitial levels of tryptophan and phenylalanine in non-depressed patients (R=0.26 and R=0.24, respectively, p<0.05), which was not present in patients with depression (p>0.1).

It is concluded that brain interstitial levels of tryptophan, phenylalanine and tyrosine are decreased in SAH patients with depression. These data support the hypothesis that the availability of neurotransmitter precursor amino acids in the central nervous system may play an important role in the pathophysiology of depressive disorders.

## FGF-23 levels and immune activation in patients with heart failure

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Cellular immune activation and disturbed iron metabolism are frequently encountered in patients with heart failure (HF). The aim of this study was to investigate the role of inflammation and iron metabolism in patients with HF and their relationship with vitamin D metabolism and heart failure parameters (NYHA stage, cardiac function, NT ProBNP).

In 149 patients (men 98, women, median age 49.8 years) with nonischaemic HF parameters of inflammation (CRP, neopterin, leukocyte counts), iron metabolism (haemoglobin, iron, transferrin, ferritin, transferrin saturation, hepcidin) and vitamin D metabolism (vitamin D, Ct-FGF-34, calcium, phosphate, PTH, klotho) were determined and their relationship with each other was analyzed.

Inflammation parameters were elevated in 72 patients (48.3 %) with HF, but no differences regarding inflammatory parameters were seen between patients with active, chronic or no myocarditis. Neopterin correlated with NT-proBNP levels and cardiac function (NYHA-class, cardiac output, right atrial pressure, pulmonary artery pressure). Neopterin and CRP were associated with each other and were significantly higher in anaemic patients. Neopterin was positively correlated with phosphate and parathormone. Ct-FGF23 levels were higher in patients with elevated neopterin and CRP concentrations, indicating that inflammation might be responsible for elevated Ct-FGF-23 levels in patients with HF.

Immune activation appears to be linked with the progression of disease, but also with alterations of vitamin D- and iron metabolism in HF. Elevated values of Ct-FGF23, which is a good predictive marker for HF, might in fact be due to immune activation.

## The structural diversity of cardiolipins and its impact on mitochondrial functions

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Phospholipids are a substantial part of the mitochondrial membrane and their composition is highly controlled and important for proper mitochondrial functioning. Besides from individual phospholipid classes especially their fatty acid side chains are important for membrane properties such as its structure, fluidity and interaction with membrane proteins. One special mitochondrial phospholipid class is cardiolipins that constitute approximately 20% of the total lipid content of the inner mitochondrial membrane, where they are almost exclusively located. Cardiolipins have a unique structure characterized by a glycerol backbone linking two phospholipid subunits thereby possessing up to four different fatty acid chains, whereby its complexity is enormous. Our group has recently established a LC/MS-MS method that allows to comprehensively quantify the cardiolipin composition, their phospholipid subunits

as well as the underlying fatty acid profile using a mathematical modeling approach.

With this method we investigated changes of cardiolipin pattern based on exogenous fatty acid uptake in cell culture experiments. HeLa cells cultured in standard growth conditions with serum as main lipid donor were compared to cells cultivated in serum and lipid free medium. The supplementation with serum resulted in severe changes of the mitochondrial cardiolipin profile. In order to change the cardiolipin pattern specifically we supplemented heart lipid extract and obtained a cardiolipin composition in cell culture mimicked the pattern of heart tissue. When analyzing the functional consequences of this rearrangement using high-resolution respirometry we found that the routine respiration of heart supplemented cells was lower than in control cells cultured in lipid-free medium although their growth rates were identical. This indicated a more efficient routine respiration. Further respirometric and biochemical experiments with permeabilized cells showed that complex I in heart lipid supplemented cells was significantly more active and could therefore be a promising candidate to explain the increased respiration efficiency.

In summary, we could show that serum in cell culture influences the cardiolipin pattern and supplementation with heart lipid extracts alters the cardiolipin profile towards mimicking heart tissue. Furthermore, these alterations lead to changes in the efficiency mitochondrial bioenergetics.

## Neopterin and anaemia are risk factors in patients with heart failure

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Anaemia and iron deficiency (ID) are frequent comorbidities in patients with heart failure (HF) and often go along with an over-activated cellular immune system. The aim of this study was to investigate the association between altered iron metabolism, vitamin D<sub>3</sub> metabolism and inflammation with disease severity and outcome of patients with heart failure (HF).

This retrospective analysis contained 149 patients (65.8 % men) with HF caused by nonischaemic cardiomyopathy and a median age of 49.8 years. The median follow-up was 58 months. By then, 19 patients had died, 5 had undergone a heart transplantation, 2 had a VAD implantation and 21 had a re-hospitalisation because of HF. Higher neopterin levels were seen in patients with more progressed HF and were predictive for an adverse outcome- independent of age, sex and established predictors in HF such as NT-proBNP, NYHA class, eGFR, anaemia, ID or Ct-FGF23. Patients with neopterin levels  $\geq 8.61$  nmol/L had a fourfold increased risk for an event compared to patients with neopterin levels  $\leq 5.70$  nmol/L. Elevated Ct-FGF23 levels were also predictive for an adverse outcome (HR 1.469 [95%CI 1.191 – 1.791],  $p < 0.001$ ), anaemia was predictive in men (HR 3.485 [95%CI 1.407 – 8.633],  $p = 0.007$ ).

Neopterin as a marker for the activated Th<sub>1</sub>-type immune system is well suited as a marker for disease severity and appears to be a good prognostic marker in patients with HF caused by non-ischaemic cardiomyopathy.

## Repetitive transcranial magnetic stimulation in patients with late life depression

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Repetitive transcranial magnetic stimulation (rTMS) is used in different neuropsychiatric conditions like Parkinson's disease, essential tremor, stroke, cognitive decline, dementia and depression. In this pilot study, safety, symptom improvement and changes in neurotransmitter precursor amino acids availability were studied after a prefrontal cortex (PFC) stimulation using rTMS as add-on treatment in ten patients with late life depression. Treatment was well tolerated and no serious adverse effects were observed. rTMS induced significant decrease in the 7-item Hamilton depression scale ( $p < 0.03$ ). The serum phenylalanine to tyrosine ratio (Phe/Tyr) declined significantly as well ( $p < 0.04$ ). These preliminary findings indicate that rTMS affects blood concentrations of neurotransmitter precursor amino acids related to neuropsychiatric symptoms in late life depression. The decrease of Phe/Tyr implies an

influence of rTMS on the activity of the primarily hepatic enzyme phenylalanine hydroxylase (PAH) expression and/or activity. PAH converts phenylalanine to tyrosine which is precursor molecule for the production of dopamine and its downstream neurotransmitters adrenaline and noradrenaline in neuroendocrine tissue [1]. Decline of Phe/Tyr thus could relate to the anti-depressive effects of rTMS therapy. However, this is a pilot study only and the finding needs to be confirmed by a larger study. Moreover, any precise mechanism how Phe/Tyr is influenced waits to be identified.

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## The blood-saliva barrier: Optimization of an *in vitro* model for the oral mucosa epithelium and its application for tryptophan transport studies

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Due to the increasing use of saliva as a diagnostic fluid in the recent years, better knowledge of the blood-saliva barrier is essential for the development of future diagnostic applications. The blood-saliva barrier consists of the epithelia of the oral cavity and the salivary glands. The oral cavity is lined by cellular layers and consists mostly of epithelial cells (keratinized or nonkeratinized in the inside of cheeks), infiltrated by other cell types, such as Merkel cells in the basal layer, Langerhans cells and Melanocytes as pigment producing cells [1]. The majority of saliva is produced by three pairs of major salivary glands (parotid, submandibular and sublingual) and approximately 10% is secreted by minor glands, which are located throughout the oral cavity [2]. Among other molecules, saliva contains proteins, nucleic acids, lipids and noncoding RNAs with varying composition reflecting the body's health. To understand the link between the concentrations of biomarker molecules in blood and saliva, comprehensive knowledge about the molecular transport mechanisms across the blood-saliva barrier is necessary.

For this purpose an *in vitro* model of the blood-saliva barrier was established using the human cell line TR146, which originated from a neck node metastasis of buccal carcinoma [3]. TR146 cells were cultivated on transwell inserts under air-lift conditions and formed a multilayered, squamous epithelia of approximately 4-7 cell layers upon cultivation for 21-45 days. For advanced growth and formation of a tight barrier different basal media and supplements were tested. Their influence on the paracellular barrier was determined by measurements of the transepithelial electrical resistance (TEER) and the permeability of carboxyfluorescein. Further characterization was conducted by qPCR using markers for keratinization (CK5, CK8), tight junctions (claudins, ZO5), as well as cornification markers. Functionality of ABC transporters were assessed using uptake assays with specific substrates. Furthermore, the cornification and growth of the cell layer were visualized by HE stainings.

As there is evidence that tryptophan might be a potential biomarker in saliva for several diseases such as bipolar disorder [4] and Alzheimer disease [5,6], the optimized model was applied to evaluate the transport of tryptophan across the blood-saliva barrier. First studies suggested that tryptophan is actively transported across TR146 cell layers into the saliva compartment. These data support a causal link between tryptophan concentrations in blood and saliva and underlines the potential of tryptophan as a possible salivary, non-invasive biomarker.

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## Spermidine and intracellular signaling

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Polyamines are small aliphatic polycations that are ubiquitously present in nature. Polyamines are involved in a variety of biological functions and processes like cell proliferation, translation, growth and aging, carcinogenesis, immune regulation. It can be suggested that some of these effects can be mediated through their ROS scavenging activity, though a specific mode of action has not yet been described for polyamines [1]. Dysbalances in polyamine concentrations are associated with pathological changes. Already decades ago it was shown that polyamine concentrations are increased in the urine of cancer patients [2] and later on, several studies reported elevated concentrations in cancer tissue and other body fluids [3]. In addition, polyamines were shown to interfere with immunological functions, and a reduction of anti-tumor activity was found in immune cells located in polyamine-rich environments [4].

This study investigated the effect of spermidine on human peripheral blood mononuclear cells (PBMC). Mitogen-stimulated PBMC can be efficiently used to investigate the immunomodulatory capacity of compounds using the biochemical pathways of tryptophan breakdown via indoleamine 2,3-dioxygenase (IDO-1) and neopterin formation via GTP-cyclohydrolase (GTP-CH-I) as readout [5]. Tryptophan and its metabolite kynurenine, as well as neopterin were measured in the supernatants of the cells via liquid chromatography and ELISA methods. Cells were treated with spermidine concentrations ranging from 1.3-10  $\mu\text{M}$  with or without mitogen stimulation, whereby concentrations of 5  $\mu\text{M}$  and below did not affect viability.

While neither tryptophan breakdown nor neopterin formation was affected by spermidine treatment in unstimulated cells, the activity of IDO-1 was efficiently reduced in stimulated cells in a dose-dependent manner, as reflected by the decreasing kynurenine to tryptophan ratio. Neopterin concentrations were not affected, though the activity of both pathway share common upstream regulators, among them most importantly interferon gamma. The preliminary analysis of upstream signaling components such as expression and phosphorylation of signal transducer and activator of transcription (STAT1) as well as of IDO-1 protein levels indicated that in the sublethal concentration range the reduction of tryptophan breakdown is due to a suppression of enzymatic activity rather than reduced expression of the enzyme.

Due to the immunoregulatory nature of IDO-1 and its involvement in tumor immune escape, the suppression of IDO-1 activity raises new questions on the role of polyamines in tumor development.

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## Oncostatin M and its receptor – overlooked members of the IL6-family

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Oncostatin M (OSM) is a member of the IL-6 family of cytokines (IL6, IL11, IL27, LIF, OSM, CNTF, CT-1, CLC) that all signal through GP130. In humans OSM induces a strong acute phase response by signaling either through the complex I of GP130 and Oncostatin M Receptor beta (OSMR $\beta$ , here used as OSMR) or through the complex II of GP130 and Leukemia Inhibitory Factor Receptor (LIFR). In an unsupervised microarray analysis of gene expression in 1 hour post-reperfusion implantation biopsies of transplanted kidneys we identified OSMR as the major inflammation-associated cytokine receptor differentiating deceased from living donor kidneys. In addition it could be shown that the severity of acute kidney injury/inflammation in renal transplant organs correlated with the expression of OSMR, outperforming the established kidney injury markers KIM1 and NGAL. Consistent with greater tissue injury, increased OSMR expression correlated with a higher risk of delayed graft function and was also a predictor of long-term graft failure in biopsies obtained at later time-points.

Based on these findings we further investigated the localization of OSMR in human kidneys and inflammation-associated cytokine transcript levels in cell cultures of human primary tubular epithelial (PTEC) and human vascular smooth muscle cells (VSMC) at baseline, under serum starvation and in response to OSM exposure.

In discarded human kidney transplants immunostaining indicated that OSMR is predominantly expressed in the renal microvasculature, in particular in smooth muscle cells. Baseline expression of inflammation-associated genes was highly different in tubular and

vascular cell lines and was in general increased due to serum starvation. OSMR induced a pronounced inflammatory response signaling via OSMR in both vascular and tubular cells, which was stronger than that seen with the other IL6-family cytokines IL6, LIF and Hyper-IL6. The effect of OSM was detectable as early as 1 hour on transcript levels of IL6, LBP, and SOCS3, was generally present by 6 hours for OSMR, SERPINA1, FGB, LIFR, and SDF1 and persisted up to 7 days for OSMR, IL6R, IL6, LIFR, LIF, SERPINA1, and FGB. The induction of genes such as LBP, SERPINA1, FGB, and LIFR underline the pro-inflammatory role of OSM, whereas the increased transcript levels of SOCS3 and SDF1 reflect an anti-inflammatory and healing response associated with OSM.

Inflammation is essential to neutralize or contain the underlying injurious process and promote the healing response. Our data indicate a key role for OSM/OSMR signaling in the modulation of pro- and anti-inflammatory responses in the kidney, both leading to inflammation and injury as well as promoting repair.

## Comfort food, addiction and the immune system

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The prevalence of obesity has almost tripled since 1975. More than 1 in 3 adults in the United States are obese. There has been an alarming increase in the prevalence of childhood obesity. Importantly, weight-loss, while being associated with decreased cardiovascular morbidity and mortality and improved cardiovascular risk, is associated with worsening of substance use (e.g. recurrence and relapse) and risk of death by suicide. This presentation details connections between appetite regulation and dysregulation, addictive behaviors, substance use disorder and immune system, to function as a platform for future research. Energy homeostasis is maintained by hypothalamic centers, which promote feeding in response to hunger signals (ghrelin), inhibit feeding in the face of long-term adiposity signals (leptin, insulin), and short

term satiety signals (CCK, distention of stomach etc.). The mesocorticolimbic (MCL) dopaminergic network, which regulates all motivated behavior, has reciprocal interactions with the hypothalamic system, such that peripheral adiposity signals, like insulin and leptin, decrease the responsiveness of MCL, while hunger signals (ghrelin) have the opposite effect. This finely regulated balance can be overridden in times of stress. Stress is associated with increasing levels of adiposity. Stress also induces preference for highly palatable (HP) foods or comfort foods, irrespective of total calorie consumption. HP foods have high density of carbohydrates or saturated fat. Stress may lead to increasing levels of adiposity due to chronically elevated glucocorticoids (GC), which in turn fail to suppress hypothalamic-pituitary (HPA) axis activation. Elevated GCs also induce preference for hedonic responses, like consumption of HP foods. GCs promote abdominal obesity and insulin/leptin resistance. Anti-stress peptides like neuropeptide-Y (NPY) promote feeding as well. HP foods have shown to buffer stress responses in both human and animal models of stress. Foods and drugs of abuse compete for overlapping mechanisms of reward regulated by the MCL dopaminergic network. Both HP foods and drugs increase dopaminergic signaling in the MCL acutely. Chronically, there is a decrease in dopaminergic (D2) receptor availability in obesity and substance use disorder. Moreover, there are overlapping endophenotypes in the realm of personality traits, neuropsychology and neuroimaging, between obesity and drug use. Both obesity and drug use have aspects of impulsivity, which later progresses to compulsive use of food and drugs. Approved weight loss medications, like Naltrexone, Topiramate, Lorcaserin and Liraglutide, have shown promise in the treatment of substance use disorders. Obesity is associated with elevated levels of acute phase reactants like C-reactive protein, as well as inflammatory cytokines like Interleukin-6, Tumor necrosis factor-alpha. HP foods directly induce insulin and leptin resistance in the hypothalamus via inflammation. Identification of inflammatory mediators in substance use disorders may unearth another dimension of overlap with obesity and pave way for novel treatment modalities.

## Inflammatory biomarkers, comorbidity and outcome in breast cancer patients

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Predictive and prognostic biomarkers play an important role in the management of breast cancer patients. The prognostic significance of inflammatory reaction that reflects the host response to neoplastic growth is being increasingly recognized. A number of biomarkers of the inflammatory response have been introduced into the management of cancer patients over the last decades, including C-reactive protein (CRP) and neopterin. Moreover, during the last few years biomarkers derived from peripheral blood cell count, including the neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR) and platelet-to-lymphocyte ratio (PLR) have been shown to represent independent prognostic biomarkers in solid tumors. In addition, all these biomarkers predict also the risk of atherosclerosis, the most important underlying cause of competitive morbidity and mortality. Early diagnosis and effective therapy result in cure in most patients affected with breast cancer, and comorbid conditions represent a competitive cause of mortality. The Charleson comorbidity index is a widely used measure to predict long-term survival based on the presence of comorbid conditions.

A retrospective analysis of a previously published cohort of patients with history of breast cancer was performed, and correlations of inflammatory biomarkers, including urinary neopterin, CRP, albumin and peripheral blood cell count-derived ratios with Charleson comorbidity index were examined. NLR, LMR and PLR were calculated from peripheral blood cell counts obtained as part of routine care. CRP and albumin have been determined by routine methods. Urinary neopterin was determined by high-performance liquid chromatography.

Urinary neopterin and serum CRP concentrations exhibited significant positive correlation with the Charleson comorbidity index, while a negative correlation was observed with albumin concentrations. However, no correlations were observed between the Charleson comorbidity index and peripheral blood cell count-derived ratios. Although history of hypertension is not included in the Charleson comorbidity index, hypertension was the most common comorbid condition in the present cohort. Urinary neopterin and serum CRP were significantly higher in patients with the history of hypertension, but no difference was observed in other inflammatory biomarkers examined. History of hypertension was also associated with significantly inferior survival.

In conclusion, urinary neopterin and serum CRP and albumin, but not peripheral blood cell count-derived ratios correlate with the Charleson comorbidity index. History of hypertension was associated with higher urinary neopterin and serum CRP concentrations, and inferior survival.

## Probing the benefits of ubiquitous phyllobilins from plants on human health - exploratory experiments in cells and possible applications

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In higher plants, the degradation of the green pigment chlorophyll furnishes linear tetrapyrroles, the phyllobilins, as major breakdown products. Phyllobilins were not only identified in senescent leaves, but also in the peels of ripening fruit and are therefore part of human nutrition [1]. Despite their versatile and interesting chemistry [2], the potential bioactivities of phyllobilins are still obscure. This study aimed at exploring the physiological roles of phyllobilins by studying their effects on cells. When investigating antioxidant activity, it was found that a phyllobilin named yellow chlorophyll catabolite (YCC) showed reactive oxygen species (ROS) scavenging activity *in vitro* and moreover in the human intestinal cell line Caco-2, indicating its bioavailability [3]. Employing Caco-2 cells to model the intestinal barrier, low micromolar concentrations of the phyllobilin were found to penetrate the cell layer while leaving the cell-cell contacts intact as investigated by immunofluorescence staining of tight and adherence junctions. To further investigate the interference with immune cells, mitogen-stimulated human peripheral blood mononuclear cells were treated with the phyllobilin, resulting a dose-dependent decrease of indoleamine 2,3-dioxygenase activity.

To summarize, data indicate that the YCC is bioavailable and suggest that this compound can get into contact with immune cells compartments due to its barrier penetrating potential where it exerts its antioxidant and anti-inflammatory properties. These experiments comprise a first clue as to the bioactivities of these ubiquitous natural

products. Further investigations will be necessary in order to deepen the studies on the uptake by and the metabolism of phylobillins in the human system.

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## Collagenase inhibitory effects of substances from marine red algae

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Exposure to UV radiation induces harmful effects to the composition of the dermal extracellular matrix (ECM), which leads to signs of photo-aging such as wrinkles, laxity and coarseness of the skin [1]. The connective tissue of the skin is altered by the reduction of collagen production and an overexpression of matrix metalloproteinases (MMPs), such as collagenases [2]. Marine algae represent an interesting source of secondary metabolites with photo-protective effects and anti-skin aging activities [3]. In this study, 12 different algae were tested for their collagenase inhibitory activity. Required extracts were obtained by consecutive extraction with dichloromethane, methanol, and methanol/water (1:1). Subsequently the extracts were fractionated, which resulted in the isolation and structural elucidation of different compounds, mainly mycosporine-like amino acids and terpenoids. All isolated compounds were investigated for their collagenase inhibitory activity and some of them showed a good dose-dependent inhibition of collagenase *in-vitro* (IC<sub>50</sub> lower than 100 µM). For deeper investigations, more MAAs, including possibly novel molecules will be isolated and tested for their biological properties focusing on photoprotective and UV-absorbing effects.

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## From adverse outcome pathways to integrated approaches to testing and assessment – for an evolution of regulatory toxicology

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Toxicological science has been increasingly focused on animal-free and mechanism-based test methods. Society and European legislation explicitly demand support for this evolution towards the realization of animal-free safety assessment. The OECD concept and program for adverse outcome pathways (AOPs) shall provide a scientific basis for this development: It aims for the identification of key events, observable *in vitro* at a cellular level, which significantly increase the chance for a toxicologically adverse outcome at organism or population level. A combination of tests which make a scientifically selected series of key events observable *in vitro* may be combined to so-called “defined approaches” (DAs) and their regulatory use may further be explained within OECD guidance for Integrated Approaches to Testing and Assessment (IATA). Skin sensitization testing is the field where this work is most advanced [1]. Key to success in further fields will be a deep uncertainty analysis of current animal-based reference methods and data. On this basis the following hypothesis can be explored: A key event related, significant effect at the cellular level may be considered as adverse, since the potential for compensation at organism level cannot be easily known due to the high complexity and variability of real life. The latter is related i.a. to variability of (epi) genetic background, preexisting disease stages, life-style, co-exposure and environmental stress. Such perspectives may also call for new GHS *in vitro* mode of action hazard classes that do not predict whatever organism level outcome, but indicate a concern as such. Clearly kinetic *in vitro* to *in vivo* dose modeling will become essential for potency differentiation. With regard to AOP, IATA and uncertainty analysis current scientific OECD work is ongoing within the field of non-genotoxic carcinogenicity [2-4]. Further work connecting basic science with regulatory applications might be stimulated by the newly founded “Austrian Platform for *In Vitro* & *In Silico* Safety Science [4].

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## Plasma and urine levels of neopterin in hemato-oncological patients and healthy controls

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Neopterin levels are elevated in body fluids during the immune system activation [1]. Neopterin could be measured in different body fluids such as blood serum or plasma, urine, cerebrospinal fluid, saliva. Elevated levels of neopterin are among the best predictors of adverse outcome in patients with HIV infection, in cardiovascular disease and in various types of cancer. Changes in neopterin concentrations in serum or urine could predict complications such as graft rejection in organ transplant recipients (e.g. bone marrow transplantation). In patient suffering from various types of malignancies, neopterin levels are often used as prognostic marker for certain types of malignancies. Neopterin levels usually correlate with the extent and activity of the disease and are useful to monitor during therapy of these patients. The aim of our pilot study was to compare neopterin levels in plasma and urine in healthy volunteers and patients with hemato-oncological disease after bone marrow transplantation. We used plasma from blood count measurement and urine from chemical urine examination. We collected 10 urine samples and 4 plasma samples during 3 weeks. Plasma and urine samples were aliquoted and stored at -80°C until analysis. Neopterin was measured by BRAHMS

ELISA (Hennigsdorf, Germany) and creatinine in urine with SIEMENS DIMENSION VISTA. We studied 8 patients (Female/Male 4/4), age (avg/median: 55/53y) and 8 healthy volunteers (Female/Male: 2/6), age (avg/median: 41/38y). Neopterin levels in plasma were 5 times higher in hemato-oncological patients (avg/median: 35/29 nmol/L) than in healthy volunteers (avg/median: 7.4/6.9 nmol/L). Moreover, neopterin/creatinine ratio in urine was significantly elevated in patients (avg/median: 488/383  $\mu\text{mol/mol}$ ) in compare to healthy volunteers (avg/median: 79/73  $\mu\text{mol/mol}$ ). Dynamics of neopterin level correlate with clinical status (outcome) of patients. The patient whose the level of neopterin was continuously increasing, has died. One patient died. Low neopterin levels (below lower than bottom limit) in healthy volunteers could be explained as better immune system or high reserve of immune system. In conclusions, neopterin represents a very promising marker for follow up of patients with hemato-oncological diseases.

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## Iron mediated suppression of CD8<sup>+</sup> T cells in a mouse mammary carcinoma model

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A dysfunction of T- and NK-cell immunity in individuals carrying mutations in iron metabolism genes has been linked to direct iron toxicity towards lymphocytes. Severe defects of the lymphopoiesis have also been described in genetic animal models of iron toxicity. In transfusion-related immunosuppression the impairment of the innate and adaptive immunity is found in patients requiring massive blood gavage to correct intra-surgery blood loss or anemia. Clinical manifestations are a higher susceptibility to bacterial infections and elevated risks of recurrence and cancer related death. Different fractions of blood preparations (leukocytes of the donor, erythrocytes, heme-bound iron) were shown to inhibit expansion and differentiation of T cells. However not much is known of the effects of impaired iron homeostasis and iron

supplementation on anti-tumor immune responses. Our preliminary results obtained in a model of mammary carcinoma in mice deficient in macrophage iron storage (ferritin H knockout mice) point towards a vicious interplay between iron supplementation, iron export from tumor-associated macrophages and a local dampening of anti-tumor cytotoxic T cells. In the proposed project we will analyse the impact of intravenous iron on the effectiveness of T-cell immunotherapies in wild-type and mutant mice.

The findings of the study will help to address safety issues on usage of iron preparations in anemic oncological individuals and will aid at selecting cancer patients for whom such treatment bears unacceptable risks. In addition, we will provide first information on the possible crosstalk of surplus iron with recently emerging immunostimulatory therapeutics like checkpoint-inhibitors and adoptive cell therapy.

## Neopterin and tryptophan metabolism in patients with seasonal Influenza infection

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Seasonal influenza is an important cause of morbidity and mortality worldwide. Neopterin (NPT), a marker of cell-mediated immune activation, is elevated in the setting of viral infections. Simultaneously, activation of the immune system via interferon-gamma results in increased tryptophan catabolism through the kynurenine pathway catalyzed by the extrahepatic indoleamine 2,3-dioxygenase (IDO). This study aimed to investigate neopterin serum levels in acute influenza infection in regard to clinical outcome parameters defined as mortality  $\leq 30$  days, ICU admission, acute cardiac events (ACE) and duration of hospitalization. Additionally, we questioned the degree of tryptophan catabolism to estimate the IDO activity.

Retrospective analysis of patients with positive influenza test, in hospital treatment of  $>24$ h and routine neopterin serum level assessment. Additionally, available

serum samples of the included patients were extracted from our biobank and neopterin, tryptophan and kynurenine concentrations, as well as the kynurenin to tryptophan ratio (Kyn/Trp), were analyzed (n=14).

30 Influenza A and 10 Influenza B patients were included in this analysis. Overall, we found an elevation of CRP and neopterin above the upper limit of the norm (ULN). Positive correlations between duration of hospitalization were found with hsTroponinT ( $r=0.45$ ,  $p<0.01$ ) and neopterin ( $r=0.37$ ,  $p=0.02$ ). Patients with ACE showed significantly higher levels of neopterin compared to those with no ACE. Significantly higher levels of neopterin ( $p<0.01$ ), kynurenine ( $p<0.01$ ), Kyn/Trp ( $p<0.01$ ) and lower levels of tryptophan ( $p<0.01$ ) were assessed when comparing Influenza patients to healthy controls.

In this study, we found a consistent elevation of neopterin serum-levels as well as a simultaneous activation of IDO as represented by an elevated Kyn/Trp in patients with acute influenza virus infection. Neopterin is strongly related to outcome parameters, and therefore it might be a helpful biomarker to rapidly recognize patients at an elevated risk of a worsened outcome.

## Changes in the tryptophan-kynurenine axis in association to therapeutic response in clinically depressed patients undergoing psychiatric rehabilitation

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In recent decades, a number of studies have shown an association between the tryptophan (Trp)-kynurenine (Kyn) axis and neuropsychiatric disorders. However, the role of the Trp-Kyn pathway on the affective status in a general psychiatric cohort requires clarification. This study aimed to measure peripheral changes in Trp, Kyn and the Kyn/Trp-ratio in individuals participating in a six-week, structured psychiatric rehabilitation program comparing subgroups of treatment responders and non-responders.

In this investigation, 87 currently depressive individuals with a life-time history of depressive disorders were divided into treatment responders (n=48) and non-responders (n=39). Responders were defined according to changes in scores of the Beck Depression Inventory (BDI-II) between time of admission and discharge (BDI-II >29 to BDI-II <14), while non-responders had no or minimal changes (BDI >20, max. 4 points difference over time). Fasting blood samples were taken and levels of Trp and Kyn, as well as Kyn/Trp and concentrations of high-sensitive C-reactive protein (hsCRP) and interleukin-6 (IL-6) were compared between groups at time of admission as well as at time of discharge.

A significant group x time interaction was found for Kyn [ $F(1,82)=5.786$ ;  $p=0.018$ ] and the Kyn/Trp ratio [ $F(1,85)=4.014$ ,  $p=0.048$ ]. Importantly, Kyn increased significantly in the non-responder group, while Kyn/Trp decreased significantly in the responder group over time. Furthermore, changes in Kyn levels correlated significantly with changes in the body mass index over time ( $r=0.235$ ,  $p=0.030$ ). No significant interactions were found for hsCRP, although levels decreased significantly over time.

Giving the limitations of the study, such as its naturalistic design and not taking into consideration psychotropic medication, we could show that the therapeutic response to a multimodal treatment in clinically depressive patients not receiving cytokine treatment is associated with changes in the Kyn/Trp ratio and Kyn levels. Treatment response was associated with reduced Kyn/Trp. Future research should clarify relevant clinical and neurobiological parameters, such as cognitive function, associated with the changes in Kyn and Kyn/Trp levels, especially in regard to clinical response.

## Immune associations with sleep, sleepiness, and seasonality of mood and behavior in the Old Order Amish

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Seasonal behavioral changes in non-human animals and seasonal affective disorder (SAD) in humans are associated with elongation of sleep duration and immune activation in winter relative to summer. We intended to measure seasonal variation in neopterin, a marker of cellular immunity, and its interactions with gender and seasonality of mood in the Old Order Amish, a population that self-forbids connection to the electric grid.

For the seasonality of neopterin, we studied 320 Amish from Lancaster, PA, USA (men = 128; 40%) with average age [SD] of 56.54 [13.93] years. Fasting blood was collected and plasma separated, aliquoted and frozen. Plasma neopterin level was measured with enzyme-linked immunosorbent assay (ELISA). Seasonality was measured with Seasonal Pattern Assessment Questionnaire (SPAQ) yielding a Global Seasonality Score (GSS) and an estimation of SAD. Statistical analysis included ANCOVAs and multivariate linear regression. We also investigated interactions of seasonal differences in neopterin with gender, seasonality scores, and estimation of SAD diagnosis. We also measured seasonal changes in sleep duration and associations between sleep impairment and sleep duration and *T. gondii* IgG antibodies, considering the role of immune system in keeping *T. gondii* in check and in altering sleep duration and other sleep parameters.

We found a significantly higher neopterin level in winter than in summer ( $p=0.006$ ). There were no significant gender or seasonality interactions. Sleep duration was longer in winter than in summer. Antibodies to *T. gondii* were not significantly associated with sleep duration, sleep onset difficulties (insomnia), but were positively associated ( $p < 0.05$ ) with sleep maintenance (improved sleep quality) and quality of wakefulness the day after sleep problems ( $p < 0.05$ ). These associations disappeared after adjustment for neopterin.

Our study confirmed the hypothesized higher neopterin level in winter, with no gender or seasonality interactions. Neopterin could be used to monitor immune status across seasons in demographically diverse samples,

even if heterogeneous in gender distribution and degree of seasonality of mood. Neopterin also seems to mediate the effect of infection with *T. gondii* on the (somewhat surprising finding) of improved sleep maintenance and problems the second day after impaired nocturnal sleep.

## Predictive associations of suicidal behavior by the synergistic interactions between TBI and inflammation-mediated medical conditions

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Inflammation mediates many aspects of the clinical and functional presentations and trajectories of Traumatic Brain Injury (TBI) and certain immune mediated clinical conditions (IMCs: infections, allergy and autoimmune). All these have been previously predictively associated, individually, with Suicidal Self Directed Violence (SSDV). We now evaluate the effect of interactions between TBI and IMCs in predicting SSDV, both lethal (suicide) and non-lethal, in a Nationwide, population-based, prospective cohort study.

All 7.22 million individuals- 15 years or older living in Denmark between January 1, 1980, and December 31, 2011, were observed during a 32-year follow-up period, with more than 149 million person-years of follow-up. Data was adjusted for sex, age, and time interval, and was analyzed with logistic regression. Individual data were drawn from Danish longitudinal registers. The relative risk for suicide and SSDV (suicide and attempts) was expressed as Incidence rate ratios (IRRs) and accompanying 95% CIs. History of TBI and infections, allergic disease and

autoimmune disease, with stratification for mental illness, were coded using ICD 8 and 10. We also analyzed SSDV using violent methods.

There was a significant interaction between TBI and IMCs in predicting SSDV- with more than additive effects for infections and allergy ( $p < 0.0001$ ), and below additive effects for autoimmune disease ( $p < 0.001$ ). The violent methods did not yield higher IRRs.

The results support the possible role of inflammation in predicting severity of TBI, mediating its SSDV risk elevating effects and perpetuating that risk. Next steps are full adjustments for possible confounders related to risk of SSDV in Denmark. If confirmed, this study may lead to studies ultimately geared to improved prognostic and interventional capabilities for preventing and managing suicidal behavior in patients with TBI.

## An attempt to theoretically predict oxidation and reduction potentials of diverse pteridine derivatives

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Pteridines represent an important and rich class of natural compounds. Due to their nitrogen-rich heterocyclic nature, chemistry of these molecules is complicated and often surprising. In recent years, several quantum chemical studies on structural stabilities of some representative compounds as well as of ionic and radical derivatives thereof were published [1-5]. All these studies employed density functional theory (DFT) level of computation and demonstrated the utility of this concept for such investigations.

Here, I report DFT studies devoted on the estimation of the oxidation and reduction potentials of pteridine derivatives. Such theoretical studies on biologically interesting molecules have become popular in recent years, and have been demonstrated to provide quite reliable results in comparison with experiments. In the case of pteridine derivatives, such investigations appear promising with regard to rare and/or short-lived structures, which are involved in important enzymatic pathways.

In principle, estimation of redox potentials involves computing the gas phase energies of a molecule and its radical cation or radical anion (one-electron reactions)

together with the relevant thermal corrections (based on frequency analysis), yielding gas phase Gibbs free energies. Further, solvation energies of the molecules and the radical ions are estimated and using the Haber-Born cycle, redox Gibbs free energies for the solvated molecules can be calculated. Dividing these by Faraday's constant, absolute redox potentials are obtained, which finally are related to the absolute standard hydrogen electrode (SHE) potential of -4.36 Volts.

We employed the B3LYP/6-311+G(2d,p) // 6-31G(d) level of theory together with a continuum approximation (the SMD model) to simulate solvent effects; all computations were done using the GAUSSIAN suite of quantum chemistry programs (Gaussian G09W, version 9.5, Gaussian Inc., Pittsburgh, PA, USA). The molecules studied were: pterin, 7,8-dihydropterin, 5,6,7,8-tetrahydropterin, 4-aminopterin, lumazine, xanthopterin, isoxanthopterin, leukopterin, and 3 tautomers of quinoid dihydropterin as model substance for important intermediates in typical enzymatic reactions involving reduced pteridine cofactors.

The computations provided quite interesting and reasonable results: when comparing, e.g., pterin with its 7,8-dihydro and 5,6,7,8-tetrahydro compounds, oxidation potentials decreased strongly with degree of hydrogenation, whereas reduction potentials increased dramatically. Thus, 5,6,7,8-tetrahydropterin was identified as the only of the studied structures yielding negative potential versus SHE when being oxidized; reduction of this molecule, however, would require an extremely negative potential.

4-Aminopterin and lumazine show somewhat higher oxidation potentials than pterin. At first sight surprisingly, oxo-derivatives xanthopterin, isoxanthopterin and leukopterin show smaller oxidation potentials than pterin, but these findings are in good agreement with earlier data on the enhancing or scavenging properties of pteridine derivatives in chloramine-T induced luminol-dependent chemoluminescence [6].

Most interestingly, quinoid dihydropterins show higher oxidation potentials but markedly lower reduction potentials as compared with 7,8-dihydropterin. This finding agrees well with the presence of quinoid dihydropterin structures in enzymatic reactions where tetrahydropterin derivatives are oxidized; the major function of the quinoid dihydropterin derivatives is being reduced again in order to regenerate the tetrahydro compound.

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## Hydroxytyrosol: the main responsible for the anti-inflammatory activity of olive

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The fruits of *Olea europaea* L. are widely consumed as a food worldwide and pits are utilized in folk medicine in order to relieve the symptoms of duodenal ulcer and gastric disturbances [1-4]. However, ingestion of olive pits may lead to gastrointestinal perforation due to the shape and the indigestible structure of the pits [5]. In the present study, we aimed to identify the possible anti-inflammatory, analgesic and antioxidant activities of aqueous extracts of black (BP) and green (GP) olive pits. Moreover, the main bioactive compound, hydroxytyrosol (HT), was isolated from olive pit and the same activity studies were performed for HT as well. The anti-inflammatory activity of extracts and HT was evaluated by measuring the level of nitrite in RAW264.7. Also, neopterin levels were measured by ELISA kit. Analgesic activity was determined by measuring prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels. According to the results, the GP extract showed significant anti-inflammatory activity in a dose-dependent manner (62.5-1000 µg/mL). Main bioactive compound HT displayed significant nitrite inhibition (84.78 ± 2.26 %) at 100 µM compared to reference compound indomethacin. Slight analgesic activity was detected only for GP extract (1000 µg/mL) and HT (100 µM). In addition, BP, GP and HT showed a significant antioxidant activity for the tested concentrations. In conclusion, these pharmacological activities are possibly related to the phenolic content of extract, mainly HT. Thus, HT might be a potential therapeutic agent for the prevention and/or treatment of inflammatory diseases. However, it is worth to emphasize that excess amounts of olive pit should not be digested because of gastrointestinal perforation risk. Therefore, a proper formulation of olive pit extract and the main bioactive compound HT might be

a potential remedy to relieve gastric disturbances related with ulcer and inflammation.

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## Alkylglycerol monooxygenase in adipocytes? An update

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Ether lipids comprise a special class of lipids which are not as well characterized as their ester counterparts. They have been shown to be essential for brain structuring, male fertility and are accumulated in fat tissues and models of adipocyte differentiation including 3T3-L1 cells [1,2]. Alkylglycerol monooxygenase (AGMO) is the only known enzyme capable of metabolizing ether lipids such as alkylglycerols and lyso-alkylglycero-phospholipids in a tetrahydrobiopterin dependent manner [3]. From our experiments we know that AGMO is expressed and active in a variety of tissues and cell lines including adipose tissue and the murine pre-adipocyte cell line 3T3-L1. However, the precise role of ether lipids and AGMO in adipose tissue is still not clear.

We used 3T3-L1 pre-adipocyte cells and manipulated AGMO activity. We studied the effect on adipocyte differentiation and looked at gene expression and protein levels of early and late adipogenic key transcription factors such as CCAAT/enhancer-binding protein  $\beta$  and

peroxisome proliferator activated receptor  $\gamma$ . In order to comprehensively study the influenced pathways, we analyzed the lipidome and transcriptome of 3T3-L1 lines with modulated AGMO activity and will report on the results.

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## RIDASCREEN® Neopterin ELISA Test

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Neopterin is a valuable biomarker of cellular immunity. Increased neopterin concentrations in body fluids such as serum and urine are associated with diseases linked with the cellular immune reaction, including inflammatory diseases, organ transplant rejections, infections and malignant diseases. To date a very limited number of commercial assays for the detection of neopterin are available on the market. We here describe the development of a new neopterin ELISA test based on microtiter plates. The assay is compatible with the use of automated laboratory systems like the Dynex® DSX. The limit of detection corresponds to approx. 1 nmol/L. Precision at the presumed cut-off level is below 10% cvd. Method comparison with a current commercial assay revealed a reasonable good agreement. Standardization of the new assay however needs to be adjusted to comply with the expected accuracy.

## In vitro testing applying human PBMC

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Freshly isolated human peripheral blood mononuclear cells (PBMC) from healthy donors can be efficiently applied as a screening model to investigate potential

immunomodulatory effects of substances, mixtures and particles [1,2]. PBMC are enriched from whole blood by density gradient centrifugation with a Ficoll-based separation medium and comprise any blood cell with a round nucleus e.g. lymphocytes, monocytes/macrophages and dendritic cells. The proportions of these populations vary across individuals, but typically PBMC are composed of 70–90% lymphocytes, 10–20% monocytes and 1–2% dendritic cells. The majority of lymphocytes are CD3<sup>+</sup> T cells (70–85%), while B cells and natural killer (NK) cells are less frequent. The population of CD3<sup>+</sup> lymphocytes is composed of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, roughly in a 2:1 ratio. After activation the CD4<sup>+</sup> T cell subset may develop into diverse effector cell subsets, including T helper (Th) type 1, Th2, Th17, Th9, Th22, follicular helper (Tfh) cells and T regulatory cells [3,4]. The different types of cell-mediated effector immunity have evolved to best respond to distinct species of microbes, viruses and insults. Further, they are characterized by the release of different cytokines, e.g. interferon- $\gamma$  (IFN- $\gamma$ ) being the most important mediator in Th1 response [5].

Besides cytokines, different stimuli can activate immune responses. Plant mitogens such as phytohemagglutinin (PHA) and concanavalin A (ConA) are used frequently in high throughput *in vitro* settings due to the stability of the response and cost effectiveness. Upon treatment of the PBMC with these mitogens, T cells release IFN- $\gamma$ , which stimulates monocyte differentiation into macrophages. In addition to its importance in defence, reactive oxygen species (ROS) release by macrophages further propagates the T-cell response.

An important pathway of the cellular immune response is the activation of the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO-1), which converts the essential amino acid tryptophan to kynurenine. Tryptophan deprivation inhibits the growth of pathogens and restricts T cell proliferation on the long-term, and tryptophan catabolites influence immune cell differentiation. The activity of IDO-1 can be expressed as the Kyn/Trp ratio [6.] The major activating cytokine for IDO-1, IFN- $\gamma$ , stimulates a variety of additional pathways, among them the formation of neopterin, a catabolic product of guanosine triphosphate (GTP) via the GTP-cyclohydrolase I in human macrophages.

Of note, accelerated tryptophan degradation and neopterin formation are associated with diseases that are characterized by cellular immune activation such as infections, autoimmune diseases, malignant disorders, and during allograft rejection periods [6]. Thus, these biomarkers are of high relevance *in vivo*. Any modulation of these immunobiochemical pathways *in vitro* may be

helpful for a first classification of immunomodulatory activity of a variety of testing agents [1,2].

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## ***Toxoplasma gondii*-oocyst IgG and symptoms of depression in the Old Order Amish**

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We recently reported that a positive association exists between “current” dysphoria/hopelessness and *Toxoplasma gondii* (*T.gondii*) serointensity. Infection with *T.gondii* may happen in multiple ways, most commonly by ingestion of either oocysts, or tissue cysts of the parasite. With their presence in contaminated water, soil and vegetables, *T.gondii* oocysts possess extreme resilience to familiar means of disinfection. Since, innovative methods to identify *T.gondii*-oocyst-specific antibodies are now available, for the first time ever; we investigated the relationships between *T.gondii*-oocyst IgG seropositivity and two cardinal symptoms of depression, i.e., dysphoria/hopelessness and anhedonia.

Data and plasma from 777 (N) Old Order Amish from Lancaster PA, with 61.4% females and a mean (SD) age of 42.4 (17.0) years. ELISA was used to assess whole plasma. *T.gondii* IgG antibodies, and *T.gondii* IgG anti-oocyst antibodies, and seropositivity was defined according to previous literature and manufacturer recommendations. Depression screening questionnaires, based on current and life-long PHQ-2, and the two analogue questions on the modified PHQ9, were also administered to the study participants part of a “Wellness” screen. Logistic regression models were used, with adjustment for age and sex, to analyze the relationship of *T.gondii*-oocyst IgG seropositivity with current and life-long symptoms of dysphoria/hopelessness and anhedonia, and a combination of both these symptoms.

A positive association ( $p=0.038$ ) was identified between *T.gondii*-oocyst IgG seropositivity and current combined dysphoria/hopelessness and anhedonia. However, relationships of *T.gondii*-oocyst IgG seropositivity with life-long anhedonia, dysphoria/hopelessness, or current symptoms in isolation, were not statistically significant. Currently, seropositivity for the *T.gondii*-oocyst IgG antibodies was significantly related to current depressive symptoms, while previously the link between *T.gondii* IgG seropositivity and current depressive symptoms did not reach the threshold for significance. This may imply that *T.gondii* oocysts, possibly secondary to a higher virulence and greater neurotropism than tissue cysts, might be able to affect to a greater degree brain substrates of mood regulation, either directly, or through immune processes and their downstream molecular mediators.

Future studies with a longitudinal design capturing reactivation or seroconversion of *T.gondii* may address some of the limitations of the current cross-sectional study, including uncertainties about the direction of causality. This would further lead to potential studies in individuals with refractory depression and high suicide risk, ultimately with preventative and treatment potential.

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## Planar chromatography in pteridines analysis

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Pteridines occur in human body fluids e.g. serum, urine, cerebrospinal fluid, saliva and play an important biological function. Among them neopterin, biopterin and their reduced forms are formed during the metabolic pathway of tetrahydrobiopterin (BH4).

The determination of pteridines is complicated and challenging due to their physicochemical properties. That is the reason, why they are usually determined by immunoassay. Among chromatographic techniques high performance liquid chromatography with fluorescence or tandem mass detection is very often chosen. Thin layer chromatography is rather rarely used [1,2].

Pteridines were separated on the chromatographic plates with silica gel 60 F<sub>254</sub> with mixture of solvents (ethyl acetate : isopropanol : 25% NH<sub>4</sub>OH, 3:4:3 v/v/v) as a mobile phase. Development tracts were linearly scanned by a TLC densitometer CS9301 equipped with a xenon lamp, to identify all pteridines by means fluorescence mode at chosen excitation and emission wavelength at  $\lambda_{exc}=370$  nm and  $\lambda_{em} > 450$  nm, respectively. This work shows how to determine together neopterin

and biopterin as well as their reduced forms by planar chromatography, after their separation from urine.

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## Alkylglycerol monooxygenase in infectious diseases

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The ether lipid cleaving enzyme alkylglycerol monooxygenase still has an almost elusive role in human physiology and pathophysiology. In cellular studies we were able to show that it is induced in murine bone-marrow derived macrophages when stimulated with anti-inflammatory cytokines whereas inflammatory cytokines priming these macrophages to an “M1” phenotype lead to a strong reduction of enzymatic activity. Knockdown of alkylglycerol monooxygenase in a macrophage cell line interferes with endogenous levels of certain lipids including the free alkylglycerols pointing to an important role of this enzyme in lipid homeostasis of the cell [1].

Among the different potential roles of alkylglycerol monooxygenase that have been recently emerging in literature from whole genome approaches, we were especially drawn to a report by Marquet et al. [2] which shows that two single nucleotide polymorphisms (SNPs) in alkylglycerol monooxygenase seem to be associated with relapses in kala-azar, a severe form of leishmaniasis, in children from a Sudanese population.

We constructed both described variants in a mammalian expression vector and analysed them for enzymatic activity and protein expression. We were able to show that one of the two variants indeed significantly affects the ability of alkylglycerol monooxygenase to catalyse ether lipid degradation whereas the second variant was functionally silent [3].

When taking into account that alkylglycerol monooxygenase is differentially regulated in macrophages, the crucial players in host defence in leishmania infection,

and a SNP associated with alkylglycerol monooxygenase impacts on enzymatic activity, this could lead to the description of a first physiological role of this lipid-cleaving enzyme in infectious diseases.

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## Fluorescence based HPLC assays for ether lipid metabolic enzymes

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Lipids containing side chains attached to the *sn*-1 position of glycerol by an ether bond instead of an ester bond occur widespread throughout the mammalian body. Their biosynthesis is initiated by three well characterized steps in peroxisomes. Further down-stream metabolic enzymes are known to occur in the endoplasmic reticulum but are less well characterized.

To allow for sensitive monitoring of enzymatic activities along the pathway we have developed assays using substrates with pyrenedecyl side chains which are readily accepted by lipid metabolic enzymes instead of natural C-16 or C-18 side chains, but contain the strongly fluorescent pyrene label. Using synthetic 1-O-pyrenedecyl-*sn*-glycerol we could set up a highly sensitive enzyme assay for the tetrahydrobiopterin-dependent alkylglycerol monooxygenase (AGMO) [1]. Substrate and product are separated by reversed-phase HPLC and the pyrene-labelled compounds quantified with fluorescence detection. The aqueous enzymatic reaction mixture is stopped with an excess of methanol and can be directly injected to the HPLC system without a lipid extraction step. This assay was essential for us to assign a sequence to this enzyme [2]. In a similar manner we have used synthetic pyrenedecanal to set up an assay for fatty aldehyde dehydrogenase [3]. We have also successfully fed pyrene-labeled compounds to intact cells to compare the

metabolism of alkylglycerol, fatty acid, fatty aldehyde and fatty alcohol [4], [5]. Using 1-O-pyrenedecyl-*sn*-glycero-3-phosphatidylethanolamine purified and processed from RAW-12, a plasmalogen desaturase deficient RAW264.7 cell line, we recently set up assays characterizing the formation of the vinyl ether bond characteristic for plasmalogens in microsomal fractions and in intact cells [6].

In summary, we find that pyrene-labeled compounds in combination with reversed phase HPLC and fluorescence detection provide a powerful tool to study the metabolism of ether lipids in microsomal preparations as well as in intact cultured cells.

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## Neopterin and tryptophan levels in patients with NSCLC treated with checkpoint-inhibitors: A prospective pilot study

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Approval of programmed cell death protein 1 (PD-1) inhibitors for non-small cell lung cancer (NSCLC) changed second line treatment for patients with metastatic disease. Although the results for PD-1 inhibitors are very promising, only 19-20 % of the patients will respond. Until yet, no established and reliable biomarker predicting response is available. In this prospective pilot study, we investigated the role of neopterin and the IDO pathway, with its metabolites tryptophan and kynurenine, as these markers have shown to be prognostic in malignancies and being valuable in measuring T-cell activation. Here, we report our preliminary results.

Serum of 23 patients with stage IV NSCLC treated in second line with PD1-inhibitors was collected prospectively at the Department of Hematology and Oncology at the Innsbruck Medical University. Specimens were drawn before treatment start, before the third application of treatment (6 weeks later) and at every restaging until progression. Neopterin levels were measured by ELISA, tryptophan- and kynurenine levels were measured by HPLC.

Twenty-three patients (47.8 % male, 52.2 % female) with a median age of 63 years were treated with nivolumab (91.3 %) or pembrolizumab (8.7 %, n = 2) as second line therapy. Patients with an objective response (13 %, n = 3) or a stable disease (26 %, n = 6) at first restaging were summarized as the “benefit” group, whereas patients with progressive disease (60 %, n = 14) were summarized as the “no-benefit” group. Mean baseline neopterin levels increased significantly between baseline and third application (17.7 vs. 32.2 nmol/L, p = 0.012) and between baseline and first restaging (17.7 vs. 31.4 nmol/L, p = 0.033). Only patients with benefit had a significant increase in mean neopterin between baseline and third application (14.7 nmol/L vs. 40.1 nmol/L, p = 0.008). No significant difference can be reported for patients with progressive disease at first restaging (p = 0.374). Baseline tryptophan levels differed between “benefit” (59.1 µmol/L) and “no-benefit” (47.2 µmol/L, p = 0.007). Both groups failed to show significant changes in tryptophan levels during the treatment. No difference in baseline kynurenine levels (p = 0.734) between groups could be shown. However, patients with benefit showed a significant increase in kynurenine between the start of therapy and third application (2.6 vs. 3.1 µmol/L, p = 0.038) and subsequently a significant decline in kynurenine till the first restaging (3.1 vs. 2.6 µmol/L, p = 0.038). No significant differences could be identified for the kynurenine/tryptophan-ratio.

Baseline tryptophan seems to be a candidate as a predictive biomarker in our study population. Further studies, including a randomized design, are necessary to proof this data.