

Pteridines  
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## Abstracts

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Organized by D.Fuchs (Innsbruck), G.Reibnegger (Graz), A. Griesmacher (Innsbruck)

### **Influence of Iron, Interferon- $\gamma$ and 1-methyl-tryptophan on Infectivity of *Chlamydia pneumoniae* within Endothelial and Monocytic Cells**

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*Chlamydia pneumoniae* is an obligatory intracellular bacterium causing acute respiratory infection and chronic inflammatory diseases in humans. The role of *C. pneumoniae* as well as the role of iron for the pathogenesis of atherosclerosis are under discussion. The influence of iron and interferon-gamma (IFN- $\gamma$ ) on immune effector pathways in *C. pneumoniae* infected endothelial cells and monocytes was investigated.

Human endothelial cells (EA.hy923) and monocytic cells (THP-1) were infected with *C. pneumoniae* and different challenges were added: iron, deferoxamine (DFO) and 1-Methyltryptophan (1-MT), the latter inhibiting the tryptophan degrading enzyme indoleamine-2,3-dioxygenase (IDO), before cells were stimulated with IFN- $\gamma$  or left untreated. The numbers of infected cells as well as inclusion bodies were counted and kynurenine, tryptophan, neopterin and tumor necrosis factor-alpha (TNF- $\alpha$ ) were determined.

In EA.hy926 cells iron supplementation and DFO promoted the infection with *Chlamydia*, while 1-MT and IFN- $\gamma$  treatment resulted in a sig-

nificant decrease of infected cells with IFN- $\gamma$  causing a more sustained reduction. In contrast, THP1 cells revealed a significant decrease of inclusion numbers with DFO and 1-MT and even more for iron. The activity of indoleamine-2,3-dioxygenase, the secretion of neopterin and TNF- $\alpha$  formation were not significantly different between iron-enriched, iron-deprived and IDO-inhibited THP-1-cells upon IFN- $\gamma$ -stimulation, respectively. In endothelial EA.hy923 cells IDO was inducible after sole infection with *Chlamydia* as well as additional stimulation with IFN- $\gamma$ . Neopterin concentrations were rather low and were unaffected by the various challenges.

The proliferation of *C. pneumoniae* is differently affected upon IDO inhibition and iron restriction between endothelial and monocytic cells. While *C. pneumoniae* within endothelial cells appears to be dependent on a sufficient exogenous supply of iron and tryptophan these pathways appear to be less important for *Chlamydia* residing within monocytes.

### **Influence of Food Preservatives Sodium sulfite, Sodium benzoate and Food Colorant curcumin on the Leptin Release of Murine Adipocytes**

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In the last decades the preparation, the preservation and at least the offer of food have led to changes in quantity and quality of food consumption. A few of different techniques were developed to preserve aliments. One widely diffused technique is the addition of antioxidants, e.g. sodium sulfite (E221), sodium benzoate (E211) and food colorants, e.g. curcumin (E100) to food. These food additives prevent the growth of bacteria and other microorganisms. Furthermore, changes of lifestyle including overnutrition and physical inactivity have led to a rise of severe obesity. Leptin, an adipocytokine, which is secreted mainly by adipocytes, is produced in proportion to fat stores. Circulating leptin serves to communicate the state of body energy repletion to the central nervous system (CNS) in order to suppress food intake. Furthermore, it is involved in the homeostasis of energy limiting the accumulation of triglycerides in liver and skeletal muscle and modulating pancreatic  $\beta$ -cells function. The aim of this study was to investigate a potential influence of food additives on the leptin release of adipocytes. We incubated murine adipocytes 3T3-L1 with the food preservatives sodium sulfite, sodium benzoate and the food colorant curcumin in various concentrations for 24 hours and measured the leptin release in the cell culture supernatant using an ELISA. We obtained a significant decrease for leptin in the cell culture supernatant after 24 hours of incubation with 1 mmol sodium sulfite ( $p < 0.01$ ). However, increasing the sodium sulfite concentration did not decrease the leptin concentration moreover. When we incubated the cells with 10 mmol sodium benzoate, we obtained a significant decrease of leptin release ( $p < 0.01$ ). When we augmented the concentration to 20 mmol sodium benzoate, we obtained a stronger decrease of leptin release in a dose dependent fashion ( $p < 0.01$ ). The food colorant curcumin decreased the leptin release significantly at a concentration of 10  $\mu\text{mol}$  and at a concentration of 50  $\mu\text{mol}$  curcumin significantly (both  $p < 0.01$ ). These data suggest that food additives affect leptin release from adipocytes. Decreased leptin release during consumption of nutrition-derived food additives would decrease the overall amount of circulating leptin to which the CNS is exposed and could therefore contribute to an adipogenic

effect.

### **Laboratory Methods for Assessing Coagulation and Platelet Aggregation in Whole Blood Samples**

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In routine platelet aggregation assays agonists are added exogenously. In routine coagulation assays plasma is highly diluted and high amounts of thromboplastin are used to trigger clotting. This obviously does not reflect the *in vivo* situation.

We therefore designed laboratory methods for assessing coagulation and platelet aggregation induced by collagen/tissue factor (TF). The clot formation process was examined by means of thrombelastometry (TEM) in whole blood (WB) triggered by addition of collagen, tissue factor, and calcium chloride. Platelet aggregation was examined by means of the impedance method in WB triggered by addition of collagen, TF, calcium chloride, and the fibrin polymerization inhibitor GPRP.

Impedance measurements show that endogenously generated thrombin (due to the addition of TF) is a potent platelet agonist. Moreover, a combination of collagen and endogenous thrombin synergistically shortens the lag time until the onset of platelet aggregation. TEM measurements allow sensitive detection of the anticoagulant action of either unfractionated heparin (UH) and low molecular weight heparins (LMWHs). For example, addition of nadroparin or enoxaparin resulted in the same dose-dependent prolongation of the coagulation time and the clot formation time, and in the same dose-dependent reduction of the maximum clot firmness and alpha angle, indicating the same anticoagulant efficacy of these two LMWHs.

Our laboratory methods allow sensitive monitoring of antithrombotic and antiplatelet actions of drugs in the physiological environment of WB.

### **Neopterin: As an Immune Marker in Gastrointestinal Cancer Patients**

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Metastasis is one of the main causes of the death in the majority of cancer patients. The spreading of the tumour to regional lymph node is often the first indicator of the dissemination of neoplasm. Development of metastasis in lymph nodes and the survival of tumour cells are related to the tumour-induced down regulation of lymph node immunity. Consequently, the lymph node situation, the progression of metastatic disease and the tumour size effects the risk of death in cancer patients. Thus, in addition to tumour biomarkers, immune system markers, such as neopterin might also be important. Although, during cancer progression, increased neopterin concentrations indicate a chronic cellular immune response, the immune system is unable to completely eliminate the stimulating agent. Hence, it is not known whether macrophages restrict their ability to recognise the cancer cells in advanced cases. Upon these findings, serum neopterin concentrations of gastrointestinal cancer patients were measured before and immediately after surgical trauma. We investigated whether the alteration of serum neopterin levels is jointly predictive with the stage of tumour, and the effect of surgical trauma is additive or alternatively, and macrophages being able to integrate multiple specific stimuli. One hundred and six consecutive cases without diabetes and immunological disorders, who underwent major-plus elective surgical interventions, were included to the study. Study designation was made according to AJCC Cancer Staging, TNM classification. Gastrointestinal

cancer patients were allocated in three groups; Primary tumour-only group (T 1-3, N0, M0; Group 2, n=28), Metastatic lymph node positive group (T 1-3, N 1-2, M0; Group 3, n=29), Locally-advanced or plus metastasis group (T4, N 1-3, M0 or any T, any N, M1; Group 4, n=11). Cancer patients' serum neopterin concentrations which were measured by ELISA (Demeditec, Germany), were compared with the serum neopterin of tumour-free individuals (Group 1, n=38). The frequency of increased serum neopterin concentrations of group 2, group 3 and group 4 were estimated by comparing with the average amount of serum neopterin of tumour-free individuals, by Mann Whitney U test. Thirty-two per cent of our control group showed higher frequency of neopterin concentrations when individually compared with their average values. In this manner, the frequencies of increased serum neopterin were 39.28 %, 41.37 %, 63.63 % for group 2, group 3 and group 4 respectively. Serum neopterin concentrations were more strongly affected by tumour invasion to adjacent tissues or distant metastases. In group 4, within the first 24-hour postoperative period serum neopterin levels simply reflected a reduced macrophage response to surgical trauma. However, in immediate post-operative period, sum of the additive frequencies of increased serum neopterin concentrations with the initial frequencies of each group were 49.99 % for group1, 64.28 % for group 2, 79.3 % for group 3 and 90.9 % for group 4. Addition of surgical trauma revealed an increasing pattern of purely additive responses in tumour-free individuals as well as in tumour bearing patients. Thus, most probably macrophages could, respond to multiple stimuli leading to increased neopterin synthesis. However, cancer patients have different concentration frequency excitement capacity in each stage despite of similar severity of operative trauma. Rising in neopterin concentration is not solely specific for tumour growth rate, but serum neopterin alterations during follow-up of cancer progression are also predictive for prognosis in one-third to two-third of gastrointestinal cancer patients.

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### **Analysis of Humoral Immune Responses in Rhesus Macaques Vaccinated with Attenuated SIVmac239Dnef and Challenged with Pathogenic SIVmac251**

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To determine the protective potential of the humoral immune response against pathogenic simian immunodeficiency virus (SIVmac251) 12 rhesus macaques were immunized with an attenuated SIVmac239Dnef either systemically or tonsillarly before challenge. Additionally, 4 unvaccinated animals were used as controls. Plasma and cell-associated viral load levels as well as neopterin concentrations were measured as disease progression parameters. Neutralizing and SIV-specific antibodies as well as complement mediated lysis were determined to characterize the humoral immune response. By in situ hybridisation trapping of viral particles was assessed in different lymphatic tissues.

Independent of the route of application, peak plasma and cell-associated viral load levels were significantly reduced by more than 2log steps in vaccinated monkeys compared to controls ( $p < 0.05$ ). Similarly, peak levels of neopterin concentrations in vaccinees were clearly reduced compared to the control cohort then. Two weeks post challenge viremia correlated indirectly with SIV-specific IgG, lysis parameters and neutralizing antibodies. During the chronic infection period 6 of 13 tested monkeys did not show any signs of trapping in lymphatic tissues. Those 6 animals had  $\leq 2$  infectious units per  $10^6$  peripheral blood mononuclear cells in the circulation then.

With this rhesus macaque study authors could demonstrate that humoral immune response

parameters and viremia revealed no significant differences in systemically or tonsillarly immunized cohorts. This fact encourages efforts for mucosal immunization strategies due to simplified application. At different time points during pathogenic SIVmac251 infection various humoral immune response mechanisms were variably active.

### **Nramp1-mediated Repression of IL-10 Formation Increases Antimicrobial Effector Mechanisms in Murine Macrophages**

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In mice, the expression of the phagolysosomal protein Nramp1 (natural resistance associated macrophage protein 1, Slc11a1) enhances host resistance to various intracellular pathogens. Nramp1 is expressed in phagocytic cells and acts as a transporter for protons, iron and other divalent cations. The expression of Nramp1 is associated with enhanced activity of pro-inflammatory pathways, including the formation of nitric oxide. Using RAW264.7 murine macrophages stably transfected with functional (RAW-37) or non-functional (RAW-21) Nramp1, we investigated the influence of Nramp1 on intracellular iron homeostasis and the production of the anti-inflammatory cytokine interleukin-10 (IL-10). We found that Nramp1 modulates the expression of diverse iron metabolism genes resulting in lower free intracellular iron levels in Nramp1-bearing RAW-37 cells. Moreover, the production of IL-10 was repressed by functional Nramp1. Upon infection of macrophages with *S. typhimurium* pathogen survival was significantly better controlled by RAW-37 cells, which correlated with enhanced formation of nitric oxide and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Addition of an anti-IL-10 antibody to RAW-21 cells resulted in increased nitric oxide / TNF- $\alpha$  production and a better killing of phagocytosed *Salmonellae*.

Furthermore, the excessive IL-10 production found in RAW-21 cells was modulated by intracellular iron availability. Taken together, Nramp1 mediates effective host defence against intracellular pathogens by suppression of excessive IL-10 production. This relates to Nramp1-mediated reduction of cellular iron pools and results in strengthening of antimicrobial effector mechanisms such as nitric oxide and TNF- $\alpha$  production.

### **Tryptophan Degradation in Cattle at risk for *Mycobacterium bovis tuberculosis***

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Bovine tuberculosis remains a significant problem in certain European countries including some parts of Austria where infection in cattle with *Mycobacterium caprae* (member of the MTB complex responsible for the tuberculosis incidents in cattle in the Tyrol) was detected recently as a reemerging disease. Diagnostic assays for bovine tuberculosis rely on the detection of specific antibodies against *Mycobacterium bovis*. *In vitro* assays which allow detection of cell mediated immune (CMI) response such as interferon- $\gamma$  production of peripheral blood mononuclear cells of cattle against specific antigens are in use for ante mortem diagnosis. In humans with *Mycobacterium tuberculosis* infections, urine and serum neopterin concentrations are increased, reflect the severity of infection and declining neopterin levels parallel successful treatment. This is true also in HIV-seropositive and seronegative adults and children with *M. tuberculosis* co-infection (1-3). However, high output neopterin production is specific for human and primate macrophages and dendritic cells stimulated with interferon- $\gamma$  but cannot be monitored in cattle. In humans, the cytokine induced neopterin production is closely associated with tryptophan degradation by indoleamine 2,3-dioxygenase, an enzyme which is induced by the same pro-inflammatory stimuli like neopterin biosynthesis (4). We therefore won-

dered whether monitoring tryptophan degradation in serum of cattle might be of any diagnostic value in cattle at risk for *M. bovis* infection.

In 100 cows from high risk *M. caprae* infection areas in the Western part of the Austrian Tyrol, blood specimens were collected for routine diagnostics tests including tuberculin skin test results, pathological scores and Bovigam test (Prionics AG, Schlieren-Zurich, Switzerland) result. Six animals presented with positive pathological findings, 11 were positive in the Bovigam test, and 25 had a positive skin test result. Tryptophan and kynurenine measurements were done by HPLC on reversed phase and monitoring the natural fluorescence of tryptophan (285 nm excitation and 365 nm emission wavelengths) and kynurenine by UV absorption at 360 nm wavelength. Average serum tryptophan concentrations resulted as  $52.4 \pm 11.1$   $\mu\text{mol/L}$ , kynurenine was  $7.7 \pm 2.4$   $\mu\text{mol/L}$  and the kyn/trp was  $157 \pm 68.7$   $\mu\text{mol/mmol}$ . Thus, tryptophan levels were considerably lower and kynurenine and kyn/trp higher than the concentrations in healthy humans: tryptophan  $73.0 \pm 14.9$   $\mu\text{mol/L}$ , kynurenine  $1.92 \pm 0.58$   $\mu\text{mol/L}$ , and kyn/trp  $26.9 \pm 8.10$   $\mu\text{mol/mmol}$  (all  $p < 0.01$ ). There was no correlation between serum tryptophan and kynurenine concentrations. When cows were grouped according to the Bovigam and skin test results and to pathological findings, positive results and pathological findings generally were associated with lower kynurenine concentrations and lower kyn/trp, whereas tryptophan concentrations did not significantly differ between groups. Kynurenine concentrations in 6 animals with pathological findings were  $6.01 \pm 1.65$  vs.  $7.84 \pm 2.45$  in pathologically normal cows. Positive results of Bovigam test were associated with lower kynurenine concentrations ( $6.14 \pm 1.27$   $\mu\text{mol/L}$ ,  $n = 11$ ) vs. animals with negative test result ( $7.70 \pm 2.54$   $\mu\text{mol/L}$ ,  $n = 76$ ;  $U = 7.79$ ,  $p = 0.02$ ). Skin test reactivity was associated with lower kynurenine concentrations ( $6.68 \pm 1.46$   $\mu\text{mol/L}$ ) vs.  $8.07 \pm 2.63$   $\mu\text{mol/L}$  in skin test negatives ( $U = 2.51$ ,  $p = 0.01$ ). In these group comparisons kyn/trp behaved similar as kynurenine concentrations. Finally, kynurenine levels correlated inversely with skin test scores ( $r_s = -0.292$ ,  $p < 0.01$ ) and pathological findings ( $r_s = -0.200$ ,  $p = 0.01$ ) as did kyn/trp but less

expressed (skin test:  $r_s = -0.226$ ,  $p = 0.01$ ; pathological findings:  $r_s = -0.152$ ,  $p = 0.06$ ). There were no such correlations between kynurenine or kyn/trp with Bovigam test results.

We conclude that *M. caprae* seropositivity/infection is associated with lower kynurenine concentrations and thus diminished tryptophan degradation compared to normal cattle. At the moment there is no good explanation for that finding, except that the enhanced nitric oxide production in cow with *M. caprae* infection may suppress IDO activity. Still, the decline of kynurenine production may support diagnosis of *M. caprae* infection in cattle.

#### References:

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- 2 Hosp M, et al. Lung 1997;175:265-275.
- 3 Horak E, et al. Lung 1998;176:337-344.
- 4 Schroecksadel K, et al. Clin Chim Acta 2006;364:82-90.

#### Neopterin concentrations in breast milk and maternal Serum and Urine

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Activation of indoleamine-2,3 dioxygenase (IDO), an enzyme converting tryptophan to N-formyl-kynurenine, was found to be a necessary requirement for immunotolerance against the fetus and thus uncomplicated pregnancy. To protect the fetus from a repulsion reaction, T cells are inhibited by expression of IDO and tryptophan degradation on the fetomaternal layer. Postpartum, plasma tryptophan concentrations were found to increase rapidly (1). In this study, plasma, urine and breast milk from 30 pregnant women were analyzed: blood and urine samples were taken three times, the day of delivery, day 1 postpartum and day 3 postpartum, breast milk samples were taken on day 2 and 3 postpartum. All patients were selected according to three dif-

ferent modi of delivery - Modus 1: women with spontaneous labor (n = 19), Modus 2: women with primary caesarean section (n = 7), Modus 3: women with secondary Caesarean section (n = 4). Neopterin was measured by ELISA (BRAHMS, Hennigsdorf/Berlin), tryptophan and kynurenine concentration were measured by reverse-phase high-performance liquid chromatography. Kynurenine to tryptophan ratio (kyn/trp) was calculated by relating concentrations of kynurenine ( $\mu\text{mol/L}$ ) to tryptophan ( $\text{mmol/L}$ ) allowing an estimate of IDO activity. The inflammation parameter C-reactive protein (CRP) was measured as well as leukocyte counts and hemoglobin levels. On day of delivery labor, parameters investigated did not differ in patients with spontaneous delivery or sections. However significant differences were observed after delivery. Patients who delivered spontaneously had significant higher tryptophan concentrations ( $p = 0.001$ ) and leukocyte counts ( $p = 0.001$ ) than women with primary or secondary caesarean section, on day 1 postpartum. Leukocyte counts increased on day 1 and decreased on day 3 postpartum, while hemoglobin levels dropped, in all groups. Neopterin plasma and breast milk concentrations did not differ between groups of delivery in longitudinal analyses. However, neopterin was detectable in breast milk while kynurenine, the first measurable product of immune-mediated tryptophan degradation could not be detected in breast milk. Tryptophan concentration in breast milk was lower on day 3 postpartum than on day 2 in patients with spontaneous delivery and secondary Caesarean section. After delivery, increasing CRP and leukocyte courses were indicative for an enhanced acute phase response, while rather stable neopterin levels indicated only a modest degree of cellular immune activation after delivery. In consequence also tryptophan degradation by IDO was only slightly activated. Interestingly no correlations existed between tryptophan concentrations in plasma and breast milk. In conclusion, our results do not indicate that enhanced immune mediated tryptophan degradation might influence tryptophan availability in the breast milk.

#### References:

- 1 Schroecksadel K, et al. Life Sciences 2003;

72: 785-793

### **Distribution of Biomarkers of Oxidation and Macrophage Activation in Human pus and Atherosclerotic Plaques**

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Plasma neopterin has been used as a key biomarker of inflammation in a number of disease pathologies (1). To date though very little analysis has been conducted on the actual site of inflammation. Our research has been investigating a number of different disease pathologies to measure the concentration of neopterin within inflammation sites as well as measuring markers of reactive oxygen species. We examined pus drainage fluid and atherosclerotic plaques removed during femoral and carotid endarterectomy.

Pus was removed by needle aspiration from 19 patients and examined for total neopterin, protein-bound DOPA, dityrosine,  $\alpha$ -tocopherol, lipid oxidation and protein carbonyls (2). Total neopterin was detected between 50 nM and 1.2 mM, with an average concentration of 0.51  $\mu$ M. Significant quantities of oxidised proteins and lipids were detected.  $\alpha$ -Tocopherol concentrations positively correlate with total neopterin levels.

Within the 16 atherosclerotic plaques analysed neopterin was found in micromolar concentrations suggesting that 7,8-DNP may significantly alter rates of extracellular lipoprotein oxidation within the plaque. The neopterin concentrations were significantly higher within the pre- and post-bifurcation region while the lipid oxidation marker, TBARS tended to be lower in these regions. The data to date shows a wide range of plaque profiles reflecting the varying levels of calcification or the presence of thrombosis.

This investigation shows that sites of inflammation could be the source of the plasma

neopterin and that the tissue concentrations may be high enough to either inhibit or promote apoptosis in different cells (1). The implications of these findings will be discussed.

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- 1 Gieseg SP, et al., Br J Pharmacol 2008; 153:627-35.
- 2 Firth CA, et al., Clin Biochem 2008;41:1078-83.

### **Inhibition of OxLDL Mediated Macrophage Necrosis by down Regulation of OxLDL Uptake, CD36 Expression and Intracellular Oxidant Production by 7,8-dihydroneopterin**

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The oxidation of low density lipoprotein (LDL) and the cytotoxicity of the resulting oxidised LDL (oxLDL) on macrophages, is considered a key part of the chronic inflammatory events leading to the formation of advanced atherosclerotic plaques. Our laboratory has shown 7,8-DNP has potent antioxidant activity *in vitro* which can inhibit both oxLDL formation and peroxy radical damage to macrophage cells (2, 3). Neopterin, an oxidation product of 7,8-dihydroneopterin (7,8 DNP) is elevated in the plasma of patients with vascular disease and is present in atherosclerotic plaques at micro molar concentrations (1, 2). In this study we have examined the protective effect of 7,8-DNP on oxLDL induced cell death in human macrophages.

Human monocytes were isolated from blood by centrifugation with Lymphoprep and differentiated into macrophages over 14 days in RPMI-1640 containing 10% human serum. OxLDL was prepared by copper oxidation from LDL prepared by ultracentrifugation of human plasma.

OxLDL induced cell death in human macrophages was inhibited by the addition of between 100 and 200  $\mu$ M 7,8 DNP. OxLDL did not trigger caspase activation but did cause a

rapid loss of cellular glutathione which was inhibited by 7,8-DNP. OxLDL induced intracellular superoxide release was found to be greatly reduced in the presence of 7,8-DNP. Analysis of oxLDL uptake using fluorescent DiI labeled oxLDL showed 7,8-DNP causing a significant reduction in oxLDL uptake over a 20 hour period. Immunoblot analyses showed there was no change in SCR-A receptor levels in the presence of 7,8-DNP but CD-36 levels were significantly reduced.

The results suggest 7,8 DNP protects human macrophages by both inhibiting oxLDL dependent generation of superoxide and macrophage oxLDL uptake. The presence of 7,8-DNP in atherosclerotic plaques may be a significant factor controlling plaque stability and growth through slowing necrotic core development.

#### References:

- 1 Gieseg SP et al., *Br J Pharmacol* 2008; 153: 627-635.
- 2 Firth C.A. et al., *Biochim Biophys Acta* 2008; 1783: 1095-1101.
- 3 Gieseg SP et al., *Free Radic Res* 2001; 35: 311-318.

### **Tryptophan Degradation and Neopterin Levels in Breast Tumor Patients**

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Indolamine 2,3-dioxygenase (IDO) is an enzyme that metabolizes tryptophan (Trp) and is expressed in a variety of cells including monocytes, macrophages and dendritic cells. IDO degrades the tryptophan to N-formyl-kynurenine which is subsequently converted to niacin as the rate limiting step in the Trp metabolism. Trp metabolism is currently under extensive investi-

gation as a possible mechanism underlying the resistance of tumor cells against cell mediated immune response. Neopterin is a worldwide accepted marker for activated cellular immune response and indirectly shows interferon gamma activated immune response. It has been detected that elevated neopterin levels or enhanced tryptophan degradation in body fluids is highly correlated with the severity of the diseases or survival data of the patients. There is not much information concerning the clinical relevance of tryptophan degradation in breast cancer patients. In our previous study we showed a significant difference in urinary neopterin levels between malignant and benign breast tumors (1). The study pointed out that neopterin levels seem to be an important and useful biomarker in diagnosis of breast tumors in clinical practice, especially in distinguishing tumor types. As a follow up study, the aim of this study was to evaluate tryptophan degradation as IDO activity and serum neopterin status in benign and malignant breast tumors. According to the results, neopterin level in the patients with malignant breast disease (n = 30) was significantly higher than benign breast patients (n = 27; p = 0.02). IDO activity did not differ significantly between malignant and benign breast disease groups, but there was a tendency towards increased activity in malignant breast disease. IDO activity significantly changed according to the grade being highest in grade III tumors (p = 0.004). Since IDO activity changes with the grade of the disease, the data indicates that IDO seems to reflect the aggressiveness of the tumor.

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- 1 Yüksel O, et al. Neopterin, catalase and superoxide dismutase in females with benign and malignant breast tumors. *Pteridines* 2007;18:132-138

### **Associations Between TIM-1-polymorphisms and Serum Neopterin Levels in Tyrolean Blood Donors**

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The TIM gene family which are found in both human and mice encodes receptors on T-cells that regulate Th1- and Th2-cell-mediated immunity. The human gene family consists of three different genes: TIM-1, TIM-3 and TIM-4. A six-amino-acid insertion at residue 157, termed 157insMTTVP (18bp insertion in exon 4) which is located at the centre of an extracellular mucin like region of TIM-1 was described to play a potential role in allergic diseases, in association with hepatitis A virus infection. The purpose of this study was to analyse potential associations between serum neopterin levels and certain genotypes of TIM-1.

Several "bi-specific" PCR using sequence specific priming (PCR-SSPs) were developed to definitively type all 4 hypothetically present haplotypes defined by 2 neighbored SNPs. Tyrolean blood donors (n = 170) were genotyped for 4 SNPs (rs1553316, rs1553318, rs12522248, rs2279804). Two coding SNPs were selected in exon 4. Another 2 SNPs were located in intron 4. Above mentioned selection of "bi-specific" PCR-SSPs allowed unambiguous detection of the 18 bp insertion in all genotypically possible combinations confirmed by direct DNA-sequencing. Four haplotypes could be identified in exon 4 of TIM-1 which resulted in 10 different genotypes.

Serum neopterin levels of 1645 blood donations in mean 9.7 donations/donor (3 - 11) were measured due to mandatory neopterin screening

Genotype TIM-1	all	<4.8		4.8 - 6.2		>6.2	
		number	%	number	%	number	%
1,1	140	34	24.3	81	57.9	25	17.9
1,2	269	56	20.8	153	56.9	60	22.3
1,4	186	66	35.5	98	52.7	22	11.8
1,3	271	65	24.0	137	50.6	69	25.5
2,2	143	37	25.9	63	44.1	43	30.1
2,4	145	63	43.4	48	33.1	34	23.4
2,3	206	65	31.6	93	45.1	48	23.3
4,4	100	8	8.0	34	34.0	58	58.0
3,4	92	18	19.6	54	58.7	20	21.7
3,3	93	23	24.7	51	54.8	19	20.4
all	1645	435	26.4	812	49.4	398	24.2

of blood donations. Neopterin screening was performed with an Elisa test. (Elitest Neopterin, Fa. Brahms).

The results of serum neopterin levels were divided into three groups, below 4.8 nmol/L (25%ile) above 6.2 nmol/L (75%ile) and between 4.8 to 6.2 nmol/L. Distribution in accordance to genotypes of TIM1 is shown in the table below.

First data show that there might be some influence of certain TIM1 genotypes on serum neopterin levels, but further investigations are needed.

### Increased Macrophage Activation as Indicated by Neopterin Levels is Associated with Poorer long-term Prognosis for Renal Aallograft Recipients

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The main determinants of long term graft and patient survival are chronic allograft dysfunction (CAD) and cardiovascular disease (CVD), disease entities characterised by inflammation and atherosclerosis. In this prospective study we analyzed the post-transplant inflammatory burden, as reflected by acute phase response and macrophage activation, in relation to long-term outcome in renal transplant patients.

In 144 (96 male, 48 female) consecutive kidney recipients the acute phase reactants serum amyloid A (SAA) and serum C-reactive protein (SCRp), the macrophage activation product neopterin in serum and urine (S/UNEOP), and SCREA were measured daily during the immediate postoperative course.

The mean period of follow up was 16 years ( $\pm$  19 months). 71 patients died with a functioning

graft, in the majority due to cardiovascular disease ( $n = 42$ ). In 19 patients the transplant failed and they returned to dialysis. The 1-, 5-, and 10 year graft survival rates were 90, 70, and 51%, the patient survival rates were 97, 77, and 59%, respectively. The mean levels of SAA, SCRP, and S/UNEOP were elevated, indicating an increased post-transplant inflammatory burden. Post-transplant SNEOP levels were significantly higher in patients with graft loss due to return to dialysis or patient death ( $p = 0.0195$ ). SNEOP and UNEOP were significantly associated with graft ( $p = 0.0403$  and  $p = 0.0209$ , resp.) and patient survival ( $p = 0.0350$  and  $p = 0.0222$ , resp.), whereas SAA, SCRP, and SCREA did not achieve statistical significance.

Early post-transplant macrophage activation, indicated by increased neopterin levels, is associated with poorer long-term graft and patient survival.

### **Central Nervous System Immune Activation is still Present after > 4 Years of Effective Antiretroviral Treatment for HIV Infection**

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HIV-1 invades the central nervous system (CNS) early in the infectious course, with detectable virus in cerebrospinal fluid (CSF). It establishes a chronic process with signs of intrathecal immunoactivation as well as detectable HIV RNA persisting in CSF, with Aids dementia as an end-stage outcome. The immunoactivation triggered by HIV can be measured as increased levels of CSF white blood cell count, neopterin and  $\beta 2$ -microglobulin, as well as increased intrathecal immunoglobulin production.

Highly active antiretroviral therapy (HAART) has led to a significant decline in HIV associated morbidity and mortality as well as in the incidence of neurologic complications. HAART

decreases signs of CSF immunoactivation markedly, but still slight ongoing intrathecal immunoactivation albeit on a lower level has been shown to be present despite otherwise effective treatment after 1-2 years. To evaluate how long time this phenomena last, we measured CSF neopterin and immunoglobulin levels in 15 virologically well treated patients for >4 years.

The median CSF neopterin concentration was 17.4 (6.7-55.3) nmol/L before treatment and 6.3 (3.7-10.7) nmol/L, and after >4 years of HAART. 9/15 patients (60 %) still had CSF neopterin levels above the upper normal reference value (5.8 nmol/L) and elevated Ig-index after >4 years of treatment. In serum the median neopterin concentration was 14.6 (8.3-37.1) before treatment and 8.0 (2.8-21.6) after >4 years treatment.

When nowadays the mortality in Aids-defining diseases almost has disappeared in parts of the world where effective treatment is available, the role of chronic inflammation in HIV infection has raised concern whether other diseases affected by inflammation such as ageing, cardiovascular disorders, dementia and non-Aids defining malignancies is a threat to long life expectancy. The clinical role of slight elevated CSF inflammation measured as CSF neopterin concentrations needs to be determined.

### **Urinary Neopterin Concentrations Show a Marked Concentration Bias Between a Commercially Available Radioimmunoassay and the HPLC Method**

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The first established urinary neopterin assay

was based on a high performance liquid chromatography (HPLC) method and since then routinely used in Innsbruck. The aim was to compare the HPLC method with a commercially available assay in our central laboratory.

Neopterin and creatinine concentrations were measured in the urine of 20 healthy, male volunteers (25 - 35y) sampled at 16 given time points during three days. Neopterin and creatinine were simultaneously determined by the HPLC method and also by a radioimmunoassay (Brahms) and the Jaffé method (Roche). Also neopterin to creatinine ratios were calculated. Assays were compared with Bland-Altman-Plots and Passing-Bablok regression analysis using MedCalc software package.

Neopterin concentrations ranged from 47 - 2696 nmol/L (HPLC) and 49 - 1787 nmol/L (radioimmunoassay) and creatinine concentrations from 0.35 - 20.71 mmol/L (HPLC) and 0.30 - 27.52 mmol/L (Jaffé). Absolute concentrations of neopterin measured by HPLC were on average 169 nmol/L higher than by radioimmunoassay, with an average bias of 29% between both assays. The regression analysis revealed a slope of 0.78 and an intercept of -8.6. In contrast, absolute creatinine concentrations were slightly lower (1.4 mmol/L) by the HPLC method than by the Jaffé method, with an average bias of 20%. Neopterin to creatinine ratios were on average 48% higher using the HPLC method compared to the methods of the central laboratory.

There is a marked neopterin concentration bias between the HPLC method and the used radioimmunoassay which has to be considered for follow-up of patients.

### **IDO - the Last Servant of Interferon- $\gamma$**

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Interferon- $\gamma$  (IFN- $\gamma$ ) has been appreciated as a prototypic pro-inflammatory cytokine linking innate and adaptive immune responses. It serves multiple biological functions mainly driving pro-

tective immune responses by augmenting the recognition and killing efficacy of phagocytosed microbes by the orchestrated actions of macrophages, natural killer cells and T lymphocytes. However, more recent evidence convincingly provides evidence, that IFN- $\gamma$  has immune down-modulating effects, e.g. it protects susceptible mice from type I diabetes or experimental autoimmune encephalomyelitis (EAE). In our studies of the immune regulatory effects of the tryptophan-metabolizing enzyme indoleamine 2,3-dioxygenase (IDO) after human HSCT we made the unexpected observation that highly activated monocytes (high neopterin secretion) also are highly sensitive to respond to stimulation with even low doses of IFN- $\gamma$  by high IDO activity, thus turning into suppressor monocytes. As presented last year we generated a model in which we showed that IFN- $\gamma$  initiated pro-inflammatory and anti-inflammatory activities in human dendritic cells (DCs) in a timely coordinated fashion and that IFN- $\gamma$  mediated IDO induction was a signature of its anti-inflammatory properties. In brief, human lipopolysaccharide (LPS)-matured DCs were exposed to IFN- $\gamma$  for either a limited (4 hours) or a prolonged (48 hours) period of time, and were subsequently used as stimulators of allogeneic T cells. While, when using cell-culture conditions optimized for proliferation, DCs generated by either activation strategy stimulated a similar proliferation of allogeneic T cells, LPS/IFN- $\gamma$  4-hour activated DCs were capable of secreting pro-inflammatory cytokines, but were IDO incompetent. In contrast, LPS/IFN- $\gamma$  48-hour activated DCs were exhausted for cytokine secretion but displayed significant and continuing IDO competence. Strikingly, the CD4<sup>+</sup>CD25<sup>+</sup> T cells retrieved from co-cultures with these DCs having been exposed to LPS/IFN- $\gamma$  for a prolonged time acquired potent suppressor activity. Thus, the anti-inflammatory effect of IFN- $\gamma$ , of which IDO activity may be a signature, and which induces regulatory activity in T cells, becomes apparent only after the IFN- $\gamma$ -stimulated production of pro-inflammatory cytokines has ceased. This is perfectly compatible with viewing the induction of IDO competence in DCs as a feed-back loop to limit potential hazardous immune reactions. Future studies will attempt to profoundly eluci-

date the mechanism underlying this timely coordinated differential effect, studying IFN- $\gamma$ R1 and IFN- $\gamma$ R2 expression and intracellular signalling.

### **Impact of Light on Circadian Rhythm in an Experimental Night Shift Setup: Neopterin, Sulphatoxymelatonin, Personal Mood and Levels of Performance**

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Shift work is a known risk factor for the development of chronic diseases like cardiovascular disorders or depression. Moreover, the constant alterations of physiological circadian patterns results in decreased quality of work, increased accident rates and absenteeism. The 24-h rhythm in our body is known to be affected by visible light especially in the short wavelength range. In our study we investigated the effects of two different lighting environments on parameters of circadian rhythm, subjective mood, and performance: Light with reduced short wavelength components (1700 K at eye level) was compared to unfiltered bright light with a higher color temperature (6300 K at eye level). For both workplaces, urinary neopterin and sulphatoxymelatonin excretion were analyzed. In addition, productivity tests and mood rating inventories were performed and the collected data was referred to the respective illumination. While nocturnal increases in aMT6-s concentrations were significantly more pronounced at night 1 and 3 of the study in the room with filtered light as compared to the room with unfiltered bright light, we could not detect any influence of the lighting environment on urinary neopterin excretion. Reducing the short wavelength components did not affect arousal, attentiveness, cognitive speed, vigilance, or subjective mood, although we could find a correlation between neopterin and the mood rating param-

eters indicative for vigour and fatigue in the room with filtered light. The maintenance of a normal nocturnal rhythm of aMT6-s without a loss in the employee's general performance may represent a benefit for employees working night shifts in office accommodations with correspondingly adapted illumination.

### **Urinary Neopterin During Therapy with Sorafenib**

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Renal cell carcinoma (RCC) is resistant to most cytotoxic agents. The cytokines interferon-alpha and interleukin-2 have only a moderate effect on the natural course of metastatic RCC. Targeted agents (multiple tyrosine kinase inhibitors sunitinib and sorafenib and anti-VEGF antibody bevacizumab) were first introduced as second line agents in patients failing cytokine therapy, but have now also significant role in the first line setting. The inhibition of tyrosine kinases involve in signal transduction may have a profound effect on immune response. We evaluated daily neopterin concentrations in patients treated with sorafenib. Urinary neopterin exhibited a correlation with disease scores.

### **Urinary Neopterin in Patients with Head and Neck Carcinoma Treated with Radiation**

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Locally advanced head and neck (HN) carcinomas can be treated in curative intent with external beam radiation (RT) alone or combined with systemic therapy (chemotherapy or biological therapy). Most of these carcinomas are histologically spinocellular carcinomas (SCC). Despite of different ways of action of oncological treatment modalities we can expect not only positive effect for the patient but also many adverse effects. The spectrum of adverse effects of radiotherapy differs from spectrum of adverse effects (AE) of chemotherapy or of biological therapy. Combination of radiotherapy with systemic therapy shows higher toxicity as single radiotherapy. We can observe acute toxicity (in the course of radiotherapy and minimal 3 month after radiotherapy) and late toxicity. High numbers of patients has severe treatment toxicity which lowers their quality of life not only in course of radiotherapy but also in their next life and very often in many years. Most common acute toxicity is - oral mucositis, radiodermatitis, xerostomy, upper gastrointestinal toxicity (dysphagia), anorexia, loss of weight, oedema of larynx etc. And late toxicity is xerostomia and fibrosis. Neopterin as a product of macrophages can serve as a marker of cellular immune system activation. Vitamin A and E are free radical scavengers and play important role in pathogenesis of oxidative stress, but they can decrease the efficacy of radiotherapy on both tumors and health tissues. Twelve patients were studied during the course of therapy. The number of urine samples ranged from 37 to 56. We correlated the level of neopterin and vitamins with toxicity of treatment.

### **Neopterin Production Correlates with Nuclear Factor- $\kappa$ B Expression in LPS-induced THP-1 Blue Cells**

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Neopterin is a well established immune activation marker and useful in several diseases associated with a Th1-type immune activation such as infections, cancer or autoimmune syndromes. In vitro, a close correlation exists between interferon- $\gamma$  (IFN- $\gamma$ ) activity, the release of reactive oxygen species (ROS) and the secretion of neopterin in human monocyte-derived macrophages and dendritic cells. Therefore, increased neopterin concentrations within a pro-inflammatory response are often associated with overwhelming release of ROS and the development of oxidative stress. In parallel, IFN- $\gamma$  induced expression of indoleamine 2,3-dioxygenase (IDO) is one of its antiproliferative strategies which is indicated by degradation of tryptophan (trp) to kynurenine (kyn). Therefore, in patients usually a close association exists between neopterin concentrations and kyn/trp, an estimate of IDO activity. The induction of signal transduction element nuclear factor  $\kappa$ B (NF- $\kappa$ B) is closely related to pro-oxidative stimuli and the translocation of NF- $\kappa$ B can be regarded as a sensitive indicator of the inflammatory response. As a consequence, NF- $\kappa$ B is deeply involved in the pro-inflammatory cascade which finally leads to the release of specific cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Therefore, an association between NF- $\kappa$ B expression and the production of neopterin and degradation of tryptophan can be assumed.

In the present study, we tested for a possible correlation between lipopolysaccharide (LPS)-induced NF- $\kappa$ B expression and the formation of neopterin and the degradation of tryptophan in monocytic cells. For this purpose, we applied the human myelomonocytic cell line THP-1-blue

which expresses a NF- $\kappa$ B inducible reporter system to study NF- $\kappa$ B activation. In cells stimulated with LPS, a significant induction of NF- $\kappa$ B was observed and this was paralleled by an increase of kynurenine and neopterin concentrations and a decline of tryptophan. Accordingly kyn/trp increased. The effects were strictly depending on the dose of LPS and on the time of incubation, higher LPS concentrations and longer incubation of cells were associated with higher activities measured. Significant correlations existed between the different biological read outs (all  $p < 0.001$ ).

We conclude that there is some evidence for a parallel induction of NF- $\kappa$ B, neopterin formation and tryptophan degradation in monocytic THP-1 cells stimulated with LPS.

### ***Crinum latifolium* L. Extracts Suppress Tryptophan Degradation in Mitogen-stimulated Peripheral Blood Mononuclear Cells**

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The plants of the genus *Crinum* (*Amaryllidaceae*) are widely used in folk medicine in different geographical regions around the world. The Indian species *Crinum latifolium* (L.) was traditionally used to treat rheumatism, fistula, tumours, earaches, rubefacient, tubercle and whitlow. In Vietnamese and Chinese traditional medicine hot aqueous extracts of *C. latifolium* (L.) are used until nowadays because of their antiviral and antitumoral activities, especially to treat prostate hyperplasia and cancer. Aqueous extracts of *C.*

*latifolium* (L.) were shown to exert significant suppressive effects on neopterin production in mitogen-stimulated peripheral blood mononuclear cells (PBMC) in vitro (1). In PBMC, neopterin production and tryptophan degradation by enzyme indoleamine 2,3-dioxygenase (IDO) are induced in parallel, and IDO was recently identified as an important antitumoral and immunoregulatory mechanism in activated monocyte-derived macrophages and dendritic cells (2).

In this study, we investigated the in vitro influence of CRILA-capsules and plant fractions C1-C3, H1, extracted from Vietnamese *Crinum latifolium* (L.), on neopterin production and tryptophan degradation in freshly isolated human PBMC. We observed significant dose-dependent effects of all five different *C. latifolium* preparations to suppress tryptophan degradation, in resting and in mitogen-stimulated PBMC. By contrast, the extracts significantly suppressed neopterin formation in stimulated cells, whereas an enhancing effect was seen in unstimulated cells. When comparing the effects of the different extracts, the extract of whole leaves always had the strongest effect on the immunobiological effects monitored. The suppression of IDO activity could represent an important aspect in the antitumoral and immunomodulatory properties of *C. latifolium* preparations in patients. It may improve the immunosuppressed state in cancer patients that seems to be closely related to the immunoregulatory role of IDO, which was found to induce regulatory T-cells. The induction of neopterin release in unstimulated cells may correspond with stimulatory effects of lectins on lymphoid and monocyte-macrophageal cell proliferation, observed earlier. In addition, direct cytotoxic effect of *C. latifolium* (L.) on tumor cells will be important, as it was demonstrated in Graffi myeloid tumor cells. Further studies are needed to characterize the effects of *C. latifolium* (L.) extracts in humans.

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### **Recombinant Expression and Purification of Fatty aldehyde dehydrogenase**

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The orphan enzyme glyceryl ether mono-oxygenase (alkyl glycerol mono-oxygenase, EC 1.14.16.5) cleaves a wide range of glyceryl ethers into glycerol and a long chain fatty aldehyde in a tetrahydrobiopterin dependent reaction. The intermediate product of this reaction, the fatty aldehyde, is then further metabolised into the corresponding acid by the long chain fatty aldehyde dehydrogenase (EC 1.2.1.48). Missense mutations in the gene coding for fatty aldehyde dehydrogenase can cause a severe disorder, the Sjögren-Larsson syndrome. Affected patients usually display less than 10% residual fatty aldehyde dehydrogenase activity compared to healthy controls. Typical Sjögren-Larsson symptoms such as ichthyosis, mental retardation and spasticity are thought to be caused by the formation of Schiff base adducts of the accumulating aldehydes with lipids or proteins containing primary amino groups.

Because of the toxic effects of fatty aldehydes, it can be assumed that glyceryl ether mono-oxygenase, whose sequence we aim to identify, and fatty aldehyde dehydrogenase act in close vicinity in the cell. To investigate this issue, we started to recombinantly express and purify fatty aldehyde dehydrogenase. The pure form of the protein will then allow us to gain further insight into the biochemical interplay between the two enzymes which would also help us in our goal to finally assign a sequence to glyceryl ether mono-oxygenase.

Fatty aldehyde dehydrogenase was expressed in *E. coli* after amplification of the correct sequence from a rat liver cDNA and subsequent cloning into a Strep-tag fusion vector. Bacterial microsomes were prepared by differential centrifugation followed by solubilisation of the mem-

brane-bound enzymes with 20% (v/v) glycerol and 0.5% (w/v) sodium cholate in order to gain an enriched raw material, which was then purified by affinity chromatography either with the combination of Strep-tag affinity and AMP sepharose column or with the combination of aminohexyl sepharose and Strep-tag affinity column. These protocols yielded 4.7% and 0.5%, respectively, of initially expressed fatty aldehyde dehydrogenase protein with an overall purification factor of 24,000 and 58,100, respectively, and a specific enzymatic activity of 27 and 54  $\mu\text{mol}/(\text{mg}\cdot\text{min})$ , respectively.

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### **Altered Immune Responses During Septicaemia in Patients Suffering from Hematological Malignancies**

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Sepsis is a life-threatening complication in patients with hematological malignancies, especially in those undergoing chemotherapy. Biochemical pathways involved in the development of septicemia and/or sepsis are only partly understood, decreased levels of mannose binding lectin (MBL) were proposed to be associated with a worse outcome in septic patients with hematological malignancies. A recent study in trauma patients on the other hand could show enhanced tryptophan degradation in non-survivors compared to survivors, indicating that over-whelming activation of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase might impair patients' outcome. In this study, we analysed markers of inflammation/immune activation including tryptophan catabolism as is reflected by the

kynurenine to tryptophan ratio (kyn/trp) and mannose binding lectin (MBL) levels towards their prognostic significance in septicemic patients suffering from hematological malignancies and "non-hematological" diseases (intensive care unit patients).

Concentrations of C-reactive protein (CRP), interleukin-6 (IL-6), MBL, neopterin, tryptophan and kynurenine were measured in sera of patients with hematological malignancies (n = 28, 36 episodes of febrile neutropenia) and in patients with sepsis on the basis of another underlying disease (n = 8) at baseline, at the onset of sepsis (as defined by high grade fever, clinical signs of systemic infection or hemodynamic instability) and during follow up.

Concentrations of CRP, IL-6 and neopterin increased significantly from baseline levels to day 1 and 3 of sepsis and decreased by day 7, while tryptophan levels decreased significantly after the onset of sepsis in patients with hematological malignancies. In "non-hematological"-septicaemic patients inflammation markers CRP, neopterin and IL-6 as well as tryptophan degradation were highest on day 1, and decreased afterwards. "Hematological patients" presented with significantly lower concentrations of neopterin and CRP on day 1, furthermore also IDO-activation as reflected by kyn/trp was lower. On the other hand, MBL levels were significantly higher in this group as compared to subjects suffering from "non-hematological" disease. Concentrations of inflammation/immune activation markers were correlated with each other, enhanced tryptophan degradation coincided with immune activation cascades. In patients suffering from hematological malignancies significantly lower MBL concentrations on day 1 and 3 were observed in patients who died.

Conclusively, septicaemic patients with hematological malignancies had an impaired pro-inflammatory immune response as compared to patients with non-hematological diseases.

### **sRANKL in Patients with HIV-1 Infection Before and During Treatment with HAART**

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Cardiovascular risk is increased in HIV seropositive patients on highly active antiretroviral therapy (HAART) but the background of this phenomenon is not yet understood. Receptor Activator for Nuclear Factor- $\kappa$ B Ligand (RANKL) is an inflammation-induced molecule which is important in bone metabolism. RANKL activates osteoclasts and overproduction of RANKL is implicated in a variety of degenerative bone diseases, such as rheumatoid arthritis. RANKL is also expressed on T helper cells and appears to be involved in dendritic cell maturation and in the regulation of T cell-dependent immune response. T cell activation was reported to induce expression of the RANKL gene and lead to an increase of osteoclastogenesis and bone loss. Serum concentrations of soluble RANKL (sRANKL) are not associated with carotid atherosclerosis but predict vascular risk (1), independently of classic vascular risk factors, like C-reactive protein or severity of carotid atherosclerosis.

In 103 patients with HIV infection (56 with AIDS), serum concentrations of RANKL were measured retrospectively by ELISA (Biomedica, Vienna, Austria) before and 12 month after HAART. Results were compared with CD4<sup>+</sup> cell counts, HIV load, urine and serum neopterin concentrations and tryptophan degradation (kynurenine to tryptophan ratio). At baseline sRANKL concentrations were  $0.208 \pm 0.258$  pmol/L, 31% of patients presenting with sRANKL concentrations below the detection limit of the assay used. sRANKL concentrations were slightly lower in AIDS as compared to non-AIDS patients (not significant). There existed significant inverse correlations between concentrations of sRANKL and virus load, urine and serum neopterin and kyn/trp (all p <0.01). HAART slightly increased

sRANKL concentrations (not significant). In parallel, CD4<sup>+</sup> cell counts increased and HIV-load, neopterin concentrations and kyn/trp decreased. sRANKL concentrations at baseline correlated significantly with levels obtained during the follow-up ( $r_s = 0.407$ ,  $p < 0.001$ ).

Our data show that a significant percentage of individuals with HIV infection presents with undetectable sRANKL, and sRANKL concentrations correlated inversely with HIV-load and several immune activation markers, probably indicating down-regulation of sRANKL during HIV infection or chronic immune activation. Whereas HIV-load and immune activation markers significantly declined during HAART, HAART seemed to increase sRANKL production in HIV seropositive individuals, albeit not significant. In HIV negative patients, higher concentrations of sRANKL were found to increase the risk of cardiovascular events (1). As angiographic data of our HIV-infected patients were unfortunately not available, it is unclear, whether presence of sRANKL may relate to plaque destabilization and rupture in HIV seropositive patients on HAART. Further studies are needed to investigate whether detectable sRANKL during HAART may indicate an increased risk of myocardial infarction.

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#### **ApoE Genotype and Neopterin Concentrations in Patients with Dementia**

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Apolipoprotein E (ApoE) polymorphisms are associated with an increase risk of Alzheimer's disease (AD). ApoE4 promotes amyloid- $\beta$  deposition and plaque formation in AD and ApoE4 allele was found to be associated with cognitive

decline and it may exert detectable effects early in the course of AD and other dementias. Earlier we have described increased serum concentrations of immune activation markers like neopterin, tryptophan degradation by indoleamine 2,3-dioxygenase (IDO) and 75kDa soluble receptor of tumor necrosis factor- $\alpha$  (sTNF-R75) in patients with progressed AD and other forms of dementias (1, 2). In this study, we measured concentrations of amyloid- $\beta$  and of tau-protein by ELISA in CSF of 94 patients (46 females, aged  $72 \pm 9.6$  years) with mild cognitive impairment or with dementias. Concentrations were compared to ApoE3 and ApoE4 polymorphisms and to serum concentrations of neopterin (measured by ELISA, BRAHMS, Hennigsdorf, Germany).

Most patients ( $n = 46$ ) presented with ApoE3/3, 18 with ApoE3/2, 30 patients had ApoE4/2-4. Between the subgroups with different ApoE alleles, no significant difference in age was recorded, but age correlated with tau-protein ( $r_s = 0.268$ ,  $p = 0.01$ ) concentrations and with CSF neopterin concentrations ( $r_s = 0.296$ ,  $p < 0.01$ ). CSF but not serum neopterin correlated with tau-protein ( $r_s = 0.611$ ,  $p < 0.001$ ). Mini mental scores and tau-protein concentrations did not differ significantly between ApoE3 and ApoE4 carriers, whereas CSF concentrations of amyloid- $\beta$  ( $p < 0.05$ ) and neopterin ( $p < 0.01$ ) as well as serum concentrations of neopterin ( $p < 0.01$ ), kynurenine ( $p < 0.001$ ), interleukin-6 ( $p < 0.05$ ) and isoprostane-8 ( $p = 0.08$ ) were lower in patients with ApoE4.

Our study shows that AD patients with ApoE4 present with lower neopterin concentrations and lower concentrations of other immune activation markers. Results confirm and extend our earlier results. On the one hand, this is a quite surprising finding because both these parameters have been found to be themselves closely related with pathogenesis of AD and other forms of dementias. On the other hand, data agree well with earlier observations by others (1) that C-reactive protein concentrations are decreased rather than increased in patients with ApoE4 allele. Our findings suggest that immune activation is another aspect which is involved in pathogenesis of dementias but is rather independent from ApoE polymorphism. Thus, immune activation appears as a risk factor for development of dementias which is rather

independent from ApoE polymorphisms.

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### **Pharmacological Modification of Iron Homeostasis by Nifedipine Affects the Course of *Salmonella typhimurium* Infection**

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Iron overload aggravates the clinical course of infections by negatively affecting cell mediated immune mechanisms of macrophages and T helper cell type 1 effector pathways. Recently, we have shown that the calcium antagonist nifedipine enhances DMT1 mediated iron transport *in vitro* and causes subsequent depletion from liver and the circulation in iron overloaded mice. Herein, we thus investigated whether nifedipine may impact on the clinical course of an invasive infection.

Mice were fed with a control diet or iron enriched diet for three weeks, infected intraperitoneally with *Salmonella typhimurium* and treated with solvent control or nifedipine for three consecutive days. Twentyfour hours later mice were sacrificed, the bacterial loads were quantified in the liver and the spleen, and the expression of iron and innate immunity genes were determined by means of RT-PCR and Western blots.

Nifedipine treated mice, independently of dietary iron overload, showed an improved survival and presented with reduced numbers of *Salmonella* in the liver and the spleen as compared to solvent injected animals. Even though these effects were more pronounced in the iron diet fed group. This was paralleled by reduced

iron content and decreased ferritin expression in the liver in the nifedipine treated group. Interestingly, we did not observe differences in the expression of a panel of pro- and anti-inflammatory cytokines according to nifedipine treatment.

Although, detrimental effects of nifedipine could have been anticipated in this sepsis model, our data provide evidence that nifedipine has a beneficial effect and may be a promising adjunct therapy by mobilizing iron and decreasing the availability of this essential nutrient for bacteria.

### **Neopterin as a Marker of Immune System Activation after Administration of Contrast Agent in Computed Tomography**

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Computed tomography (CT) is one of the most widely used and important diagnostic methods. The CT examinations are done in three phases; a native examination, arterial contrast phase and portal - venous contrast phase. Contrast agent is an iodine solution which is administered intravenously that helps in demarking blood vessels and soft tissues, pelvic abdominal and chest organs and pathological formations, e.g. malignant tumors. The contrast medium can cause an allergic reaction from skin changes to anaphylactic reaction. Because of allergic reactions, all patients must be pre-medicated with antihistamine agent.

**Homocysteine Concentrations in Patients with Metastatic Colorectal Carcinoma During Combined Therapy with Bevacizumab, Oxaliplatin, 5-fluorouracil and Leucovorin**

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The prognosis of metastatic colorectal carcinoma has improved substantially during the last decade as a result of introduction of new effective cytotoxic agents and, more recently, biological agents, the monoclonal antibodies cetuximab and bevacizumab. It has been demonstrated that combination of 5-fluorouracil with either irinotecan or oxaliplatin is, in terms of efficacy, superior to 5-fluorouracil alone. The addition of bevacizumab, monoclonal antibody against vascular endothelial growth factor (VEGF), to combination chemotherapy has been shown to increase survival both in the first line as well as in the second line setting. Although the therapy with bevacizumab is usually well tolerated, the drug has a peculiar toxicity profile with the principal side effects including proteinuria, hypertension and thrombosis. These toxicities may be of concern in long-term survivors because they are associated with increased risk of atherosclerosis. In addition, VEGF is an endothelial growth factor and VEGF blockade leads to endothelial cell dysfunction.

Homocysteine is an intermediate in the metabolism of methionin. Increased serum concentration of homocysteine is, in most cases, caused by a deficiency of folate or vitamin B12. Hyperhomocysteinemia has been amply documented in patients with vascular disorders, and increase homocysteine concentrations are a well defined risk factor of atherosclerosis and thrombosis. Hyperhomocysteinemia has also been documented in cancer patients. The aim of the present study was to evaluate the effect of combination therapy with bevacizumab, oxaliplatin, 5-flu-

orouracil and leucovorin on serum homocystein in patients with metastatic colorectal carcinoma.

**Effect of Therapy with Aromatase Inhibitors on Urinary Neopterin, Serum Homocysteine and C-reactive Protein in Patients with Breast Carcinoma**

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Aromatase inhibitors are effective hormonal therapy in patients with early or advanced/metastatic breast carcinoma. By suppressing endogenous estrogens aromatase inhibitors may affect lipid metabolism, inflammatory response and antioxidant balance. One-hundred-eighty six post-menopausal patients with histologically verified breast carcinoma were studied immediately before as well as 2 and 4 months after the start of therapy with aromatase inhibitors. A significant increase in C-reactive protein was observed 2 and 4 months after the start of therapy with aromatase inhibitors, but no change in neopterin or homocysteine was noted. Patients treated with tamoxifen had also lower serum C-reactive protein and urinary neopterin levels.

**Asymmetric dimethylarginine and Antioxidants in Patients with and without Angiographic Coronary Artery Disease**

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Asymmetric dimethylarginine (ADMA), a

competitive inhibitor of the nitric oxide synthases, is produced by methylation of arginine residues in intracellular proteins by the enzyme arginine N-methyltransferase. Elevation of circulating ADMA levels leads to an increased vascular tone and blood pressure and plays a pivotal role in endothelial dysfunction and thus seems to be important in the pathogenesis of cardiovascular disease (1, 2).

In order to investigate ADMA metabolism, in a cross-sectional approach, blood concentrations of ADMA, antioxidants, homocysteine, neopterin, folic acid and vitamins B6 and B12 were compared in 1477 patients, which were recruited within the Ludwigshafen Risk and Cardiovascular Health (LURIC) study.

ADMA concentrations were not significantly different in patients with coronary artery disease (mean  $\pm$  SD:  $0.81 \pm 0.14$   $\mu$ mol/L) and controls ( $0.81 \pm 0.15$   $\mu$ mol/L; Welch's t test:  $p = \text{n.s.}$ ). On the other hand, there were significantly lower concentrations of  $\gamma$ -tocopherol, ascorbic acid,  $\alpha$ -carotene,  $\beta$ -carotene, lycopene and lutein/zeaxanthin (all  $p < 0.05$ ) but not of  $\alpha$ -tocopherol in coronary artery disease. Patients with coronary artery disease had higher homocysteine (mean  $\pm$  SD:  $13.9 \pm 6.1$   $\mu$ mol/L) and neopterin ( $8.7 \pm 7.2$  nmol/L) concentrations compared to controls (homocysteine:  $12.7 \pm 5.3$   $\mu$ mol/L; neopterin:  $7.4 \pm 5.0$  nmol/L; both  $p < 0.001$ , Welch's t test). There were significant inverse correlations between ADMA concentrations and levels of vitamin B6 ( $r_s = -0.183$ ;  $p < 0.0001$ ), and levels of antioxidant compounds  $\alpha$ -tocopherol ( $r_s = -0.068$ ,  $p < 0.01$ ), ascorbic acid ( $r_s = -0.061$ ,  $p < 0.05$ ),  $\alpha$ -carotene ( $r_s = -0.113$ ), lycopene ( $r_s = -0.180$ ), and lutein/zeaxanthin ( $r_s = -0.107$ , all  $p < 0.0001$ ). Finally, ADMA correlated positively with homocysteine ( $r_s = 0.226$ ) and neopterin concentrations ( $r_s = 0.286$ ; both  $p < 0.0001$ ). In a multiple regression analysis the variables,  $\alpha$ -tocopherol and lycopene significantly contributed to the model indicating the independent influence of these variables on ADMA concentrations.

Data show an association of higher ADMA production with a decline of antioxidant compounds and vitamins and a concomitant increase of homocysteine and neopterin production in patients at risk for atherosclerosis. The inverse

relationship between diminished concentrations of antioxidant compounds and ADMA implies an influence of oxidative stress to increase ADMA levels most probable by a reduced activity of dimethylarginine dimethylaminohydrolase (DD-AH). This enzyme cleaves ADMA to form dimethylamine and citrulline and its expression is supposed to be down-regulated by oxidative stress (2). Data would be in line with the assumption that the increase of ADMA is secondary to the chronic inflammation and immune activation which is associated with cardiovascular disease. It corresponds with the significant correlation found between ADMA and neopterin concentrations and these with the fact that an increased ADMA production is also found in stimulated peripheral blood mononuclear cells (3).

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#### **The hemochromatosis-associated Hfe mutation protects mice from Salmonella typhimurium infection via induction of lipocalin 2**

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Mutations in HFE predispose to hereditary hemochromatosis type I, a frequent genetic disorder characterized by progressive parenchymal iron deposition and eventual organ failure. Since

HFE mutations are associated with reduced iron levels within mononuclear phagocytes, we hypothesized that Hfe deficiency may be beneficial in infections with intramacrophage pathogens.

Using *Hfe*<sup>+/+</sup>, *Hfe*<sup>+/-</sup> and *Hfe*<sup>-/-</sup> mice in a model of typhoid fever, we found that animals lacking one or both Hfe alleles are protected from systemic infection with *Salmonella typhimurium*, displaying prolonged survival and improved bacterial control. This increased resistance can be referred to an enhanced production of the siderophore-binding peptide lipocalin 2 and the reduced availability of iron for *Salmonella* engulfed by Hfe deficient macrophages. This effect is mediated via stimulation of lipocalin 2-dependent iron export from infected cells since *Hfe*<sup>-/-</sup> macrophages concurrently knocked out for lipocalin 2 are unable to efficiently control the infection or to withhold iron from intracellular *Salmonella*. Correspondingly, infection of *Hfe*<sup>+/+</sup> and *Hfe*<sup>-/-</sup> mice with siderophore deficient *Salmonella* abolishes the protection conferred by the Hfe defect.

Thus, by inducing the formation of the iron-capturing peptide lipocalin 2, the Hfe mutation harbors a genetically determined immunological advantage towards infections with intracellular pathogens such as *Salmonella*.

### **Serum Phenylalanine Concentrations in Patients post Trauma and Burn Correlate to Neopterin Concentrations**

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Aromatic amino acid phenylalanine (Phe) is essential for humans and precursor for tyrosine (Tyr), which is another important amino acid within the biosynthesis of hormones and neurotransmitters like DOPA and catecholamines dopamine, epinephrine and norepinephrine (1). Increased blood levels of Phe have been already reported earlier in patients with HIV infection,

cancer, trauma, sepsis and burn (2-4). All these clinical conditions are known to be linked with inflammation and immune activation and with increased concentrations of neopterin. In a small number of patients after trauma, a correlation between neopterin and Phe concentrations and Phe to Tyr ratio (Phe/Tyr) was described recently (5).

In this study, Phe, Tyr and neopterin concentrations were measured in 20 patients post trauma, in 31 burned patients and in 30 healthy controls. Patients' sera were collected during one week of follow-up. Phe and Tyr concentrations were measured simultaneously by fluorescence detection after reversed phase HPLC and neopterin concentrations by ELISA (BRAHMS, Hennigsdorf, Germany). At the begin, Phe and Tyr were subnormal in both patients' groups, but during follow-up, average concentrations of Phe and neopterin and Phe/Tyr were increased in patients post-trauma and burns compared to healthy controls, Tyr concentrations were decreased. A significant correlation existed between neopterin and Phe concentrations and Phe/Tyr in the trauma and in burned patients.

All the patients received parenteral nutrition, so a different dietary intake of Phe or Tyr cannot underlie differences in the increases of Phe and Phe/Tyr between patients during follow-up. Rather a biochemical alteration in the conversation rate of Phe to Tyr by enzyme phenylalanine-4-hydroxylase (PAH) seems to be involved. An impaired PAH activity could be due to insufficient supply with cofactor 5,6,7,8-tetrahydrobiopterin (H4Bip) which is required as a hydrogen donor within the enzymatic hydroxylation reaction (1). H4Bip is very sensitive to oxidative stress and in conditions of cellular immune activation, when oxidative stress develops, H4Bip can be depleted and PAH activity declines because of insufficient cofactor supply. Thus, the correlation found between increased Phe and Phe/Tyr and neopterin concentrations in trauma and burned patients implies that the activated cellular immune system is involved in the reduced hydroxylation of Phe. However, in our study we found such a significant correlation relationship even in 30 healthy controls (neopterin vs. Phe:  $r_s = 0.415$ ;  $p < 0.01$ ; vs. Phe/Tyr:  $r_s = 0.465$ ;  $p < 0.01$ ). This finding sug-

gests a rather general association between diminished PAH activity and immune activation.

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### **Cofactor Rather than Antioxidative Activity of Tetrahydrobiopterin Prevents Ischemia Reperfusion Injury Following Murine Pancreas Transplantation**

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Tetrahydrobiopterin (H<sub>4</sub>B) is both, an essential cofactor of nitric oxide synthases (NOS), and thus a critical determinant of nitric oxide (NO) production, and a strong antioxidant. Here we compare the recently shown protective effect of H<sub>4</sub>B on ischemia reperfusion injury (IRI) following murine pancreas transplantation with tetrahydroneopterin (H<sub>4</sub>N), a pteridin analogue with similar chemical redox behaviour but no cofactor activity, and with the antioxidant vitamine C (VitC) in order to evaluate the importance of the antioxidative activity.

Male syngeneic C57BL6 mice were used as size-matched donor and recipient pairs. Cervical heterotopic vascularized pancreas transplantation was performed with a modified no-touch technique. Pancreatic grafts were subjected to 16h prolonged cold ischemia time (CIT) as well as 45 minutes warm ischemia time (WIT) and different treatment regiments: untreated animals (I), H<sub>4</sub>B

50mg/kg b.w. i.m. prior to organ retrieval (II), H<sub>4</sub>N 50mg/kg b.w. i.m. prior to organ retrieval (III) and VitC 350mg/kg b.w. i.m. prior to organ retrieval (IV). Non transplanted animals served as controls (V). After 2h of reperfusion intravital fluorescence microscopy was used for analysis of graft microcirculation by means of functional capillary density (FCD). Quantitative assessment of parenchymal damage was analyzed by histology (H&E) and by performing nitrotyrosine-immunostaining to determine peroxynitrite formation.

Following prolonged CIT only pancreatic grafts treated with H<sub>4</sub>B prior to retrieval (II) displayed markedly higher values of FCD compared to non treated animals (I) (p <0.01). In contrast, neither pre-treatment of donor animals with H<sub>4</sub>N (III) nor with VitC (IV) did improve FCD. Compared to non treated animals application of both pteridin analogues as well as VitC significantly attenuated parenchymal damage as well as peroxynitrite formation (p <0.05). However, reduction of early parenchymal damage in pancreatic grafts was clearly more pronounced by H<sub>4</sub>B pre-treatment compared to the other two treatment groups (p <0.05).

The protective effect of H<sub>4</sub>B on ischemia reperfusion injury relies on its NOS cofactor activity rather than on its antioxidative capacity and provides a new therapeutic target in the prevention of IRI following solid organ transplantation.

### **Oxidative Modified Human Serum Albumin: New Aspects of a well Known Molecule**

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Human serum albumin is the main protein in plasma and has a number of functions, predominantly transport of endogenous and exogenous

compounds. During its average lifetime of 27 days albumin is prone to a number of modifications. These include reactions with glucose, hydrogen peroxide or hypochlorous acid. Modification of albumin may be determined in different ways: The redox state of cysteine-34 which is not involved in an intramolecular disulfide bond, the content of carbonyl groups in the molecule and the content of advanced oxidation protein products were the parameters of our choice.

We found that during exercise the thiol form of cysteine-34 in albumin was decreased, depending on exercise intensity. This effect was significant and reversible. Supplementation with fruit and vegetable extract had no effect. In liver disease albumin is severely oxidized indicated by an increase in carbonyl groups as well as an oxidation of cysteine-34. In parallel, the binding properties of albumin for bilirubin and dansylsarcosine are impaired in liver disease patients. *In vitro* oxidation of albumin by hypochlorous acid leads to an increase in carbonyl groups and advanced oxidation protein products on albumin. In addition, oxidation converts albumin to a potent ligand of the hepatic scavenger receptor BI. Albumin oxidized by HOCl inhibited binding of HDL to its receptor *in vitro* and decreased clearance of HDL *in vivo*. Albumin isolated from hemodialysis patients showed the same effects while albumin from healthy donors did not.

Oxidative modification of albumin results in an impairment of its physiological functions and in addition in the occurrence of new, pathophysiologically relevant properties.

### **Tryptophan Degradation, Neopterin and Nitrite/Nitrate Concentrations in Serum of Children with Malignant Diseases**

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Neopterin represents a sensitive indicator of cellular (=Th1-type) immune response. The determination of neopterin was demonstrated to be useful for predicting prognosis in HIV-1 infections and in coronary artery diseases and also in various types of cancer. However, it is interesting to note that in more than 400 papers published on neopterin in cancer up to now, there is only one article on neopterin as a laboratory diagnostic tool in childhood cancer (1). In adult patients with cancer, usually a close correlation is found between neopterin production and tryptophan degradation by indoleamine 2,3-dioxygenase (IDO) (2), the kynurenine to tryptophan ratio (kyn/trp) allowing an estimate of IDO activity.

This pilot study investigates 9 children (2 females) with cancer (average age 9.0 y, range: 2-18 y; 2 females; 4 patients with leukemia, 1 non-Hodgkin lymphoma, 4 solid tumors at diagnosis and during several weeks of follow-up. In addition, 10 controls (average age 9.7 y, range: 2-18 y; 5 benign tumors, 5 malignancies in remission, 2-4 y after therapy) were investigated. Serum neopterin concentrations were measured by ELISA (BRAHMS, Hennigsdorf, Germany), and in addition tryptophan and kynurenine were determined by HPLC, nitrite/nitrate by Griess reaction, and procalcitonin (PCT) (Kryptor, BRAHMS).

Serum neopterin concentrations were higher in children with malignant diseases ( $p < 0.05$ ), this was especially true in the children with leukemia and lymphoma ( $p < 0.01$ ). Also tryptophan degradation was increased in children with malignant disease, tryptophan levels were significantly lowered ( $p < 0.05$ ), whereas the variation of nitrite/nitrate and PCT concentrations was too wide for a statistically significant result in group comparisons. During follow-up, higher neopterin concentrations and kyn/trp were observed preferentially in non-survivors as compared with survivors. The significant correlation between kyn/trp and neopterin ( $r_s = 0.757$ ,  $p < 0.001$ ; calculating all data including follow-up) indicates parallel activation of neopterin production and tryptophan degradation by IDO in the children with cancer. Also PCT levels correlated with kyn/trp and neopterin, but to a lesser degree (kyn/trp:  $r_s = 0.437$ , neopterin:  $r_s = 0.369$ ; both  $p < 0.001$ ). Inverse associations existed between ni-

trite/nitrate and neopterin ( $r_s = -0.296$ ) and kyn/trp ( $-0.356$ , both  $p < 0.001$ ). The latter data would agree with an inhibitory effect of NO production on IDO expression and activity.

Results of this pilot study indicate that monitoring of neopterin production and tryptophan degradation in children with cancer may reveal useful information for diagnosis and prognosis. However, due to the so far small number of patients studied, further studies are needed to confirm this conclusion.

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### **Anti-inflammatory Effects of Colostrum**

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Bovine colostrum is the thick yellow fluid, which a lactating cow gives to a suckling calf during its first days of life. Bovine colostrum is rich in growth factors, immunoglobulins, and antimicrobial factors and also contains abundant nutrients such as carbohydrates, fat, vitamins and minerals. The biological function of colostrum is to support the growth of the calf and prevent gastrointestinal infections until the calf has synthesized its own active immune defense system. The immune system of the newborn is stimulated by colostrum, which increases the rate of maturation of B-lymphocytes. Colostrum contains 1.5-5 g/L lactoferrin, an 80 K protein that has been shown to transport essential iron to haematopoietic cells and to prevent harmful viruses and bacteria from getting the iron they need for growth. Besides the antimicrobial activity of lactoferrin, lactoferrin may play an important role in iron uptake in the intestine and in the activation of phagocytes and immune responses. Although colostrum has only

received widespread attention as a dietary supplement since the late 1990s, it has a long history of medicinal use. Colostrum is currently also used in a topical cream for immunorelated skin problems like atopic dermatitis or psoriasis. However, despite a large amount of literature concerning the properties of human or bovine colostrum, there is only sparse data on the effects of colostrum on the human immune system.

In an approach to evaluate the effects of bovine colostrum on the T-cell/macrophage interplay, we investigated the capacity of various colostrum nanoparticle preparations to modulate tryptophan degradation and formation of neopterin in unstimulated and phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMC). The results were compared with the effects of lactoferrin and the globally accepted cosmetic preservative Euxyl 9010, which was used in creams to preserve the colostrum ingredients.

In unstimulated PBMC, all colostrum preparations stimulated tryptophan degradation and formation of neopterin. By contrast colostrum suppressed these biochemical activities in PHA-induced PBMC. There was no influence of lactose content on the activity of colostrum, but pure lactoferrin suppressed IDO activity and induced only a slight increase of neopterin in the supernatant of unstimulated cells. Lactoferrin suppressed both these pathways in PHA-treated cells. Euxyl enhanced the suppressive activity of colostrum and attenuated the stimulatory potential.

The finding that colostrum stimulates neopterin production and tryptophan degradation in unstimulated PBMC implies that it is able to stimulate Th1-type immune response and thereby may slow-down Th2-type immune activation which could relate to beneficial effects of colostrum to treat atopic dermatitis: However, colostrum was also found to counteract activation cascades in mitogen-stimulated PBMC, and these activities might rather relate to the therapeutic effects seen in patients with psoriasis. Also lactoferrin had similar potential as colostrum to suppress PBMC stimulation, albeit the effect is much smaller at even higher concentration. Lactose content only slightly modulated activity of colostrum. Euxyl

was found to enhance the suppressive activity of colostrum on PBMC and it slows-down its stimulatory potential, however only at much higher concentrations than those present in the colostrum preparations used in this study. Overall, data show significant immunomodulatory effects of colostrum preparations which might relate to at least some of its therapeutic usefulness. Effects appear to be independent from lactoferrin or lactose content.

### **Inflammation, Mood Disorders and Suicide**

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Certain Th1-type cytokines activate and certain Th2-type cytokines inhibit indoleamine 2,3-dioxygenase (IDO) which shifts tryptophan metabolism from serotonin synthesis toward the kynurenine pathway tryptophan availability is the rate-limiting step in the synthesis of serotonin, and its reduced availability is associated with the induction of a depressive relapse in vulnerable patients. Serotonin abnormalities have been implicated in recurrent depression, anxiety, impulsivity, suicidality and completed suicide (1). It has been consistently shown in patients with cancer and hepatitis, treatment with interferon- $\gamma$  or interleukin-2 is associated with syndromes depression, significant decreases in tryptophan, increases in kynurenine, and decreases in the kynurenine/tryptophan ratios (2). Moreover, the decrease in tryptophan, the increase in kynurenine, and the decrease in tryptophan/kynurenine ratio have been correlated with the severity of depression (3). Antidepressant treatment attenuates interferon- $\gamma$  induced depressive symptoms (4). We will briefly review the literature and present our own results associating airway inflammation (predominantly Th1 or Th2), symptoms in recurrent mood disorders, suicide risk factors and

completed suicide. In one study recently completed, although kynurenine/tryptophan levels at baseline predicted emergence of suicidal ideation in patients with mood disorders exposed to environmental levels of tree pollen, the association between changes in kynurenine/tryptophan and suicide ideation was negative rather than positive. This is consistent with a previously reported lower, rather than higher activity of IDO activity in atopic individuals (5), and suggests that activation of IDO does not mediate the association between allergy and affective/behavioral abnormalities. There is a need for multilevel inquiry (epidemiological, clinical, animal, imaging, post-mortem) to advance the emerging field of affective neuroimmunology, and uncover novel therapeutic targets in mood disorders and suicide prevention.

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### **Purification and Characterization of Unexplored Enzyme Pterin Deaminase from Native Isolate *Micrococcus luteus* B1252**

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Antifolates are class of antimetabolite drugs widely used in cancer therapy. Asparaginase is an anti-neoplastic agent, used in lymphoblastic leukemia chemotherapy. Similarly, the most

unexplored and possibly a potent drug is the enzyme pterin deaminase, which deaminates folic acid to products 2-hydroxy-2-deamino pteroyl glutamic acid and ammonia. The deaminated product could not be utilized for the DNA synthesis as it differs structurally which results in folate deficiency, thus reduces the proliferation of cancerous cells. In January 1976, U.S. Patent No. 3,930,955 was awarded to Kusakabe et al. for the process of producing pterin deaminase having antitumour activity.

In spite of the patent and exploration of the preliminary antitumour property of enzyme pterin deaminase and its various roles in prokaryotes and eukaryotes, very few works has been carried out in elucidating the biochemical properties of the enzyme. The reasons behind the lack of work are unclear currently and this tempted us to speculate and investigate the properties of pterin deaminase from *Micrococcus luteus* BI252.

In the present study, we have isolated and characterized the enzyme pterin deaminase from native isolate *Micrococcus luteus* BI252 (Accession number: EU176162). The enzyme was partially purified in a sequence by precipitation and ion-exchange chromatography. The purification of the purified pterin deaminase increased 15 fold over that of crude and exhibited maximum activity of 3.280U/mL at the optimal conditions (pH 8.5 and temperature 30°C). Km and Vmax respectively were found to be 24 mM and 3.175U/mg against substrate folic acid. On SDS PAGE, the molecular weight of enzyme was found to be 40 KDa. Product analysis and activity staining of the purified enzyme was confirmed respectively on thin layer chromatography and Native PAGE based on the fluorescence property of product. The enzyme does not exist in isoform and has isoelectric point of 6.3. Among various inhibitors tested for their effect on enzyme activity lumazine at 1µM concentration was found to inhibit almost completely having residual activity of 0.2%. The antitumour property of the enzyme was studied on two human breast cancer cell lines MCF-7 and MDAB231. Cell death induced by apoptosis was observed in MCF-7 cell lines were as no such effect was observed in MDAB231 cell lines. At 350µg/mL enzyme concentration 50% inhibition was observed in MCF-7 cell lines.

Thus the present work renovate the basic study of characterizing the enzyme pterin deaminase which would lay a foundation and fling light for further exploration for cancer therapy.

### **Biological Implications of Unexplored Enzyme Pterin Deaminase**

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Antifolates are widely used drugs in cancer therapy that inhibit folate dependent enzymes and exert a powerful impact on folate dependent cell division resulting in inhibition of proliferation leading to cell death. Numerous studies have demonstrated that despite targeting multiple metabolic pathways, antifolates ultimately kill cells by induction of biochemical cascades associated with apoptosis. The molecular mechanisms that connect folate metabolism and induction of apoptosis are not clear at present. Most antifolates kill cells in S phase of the cell cycle as duplication in the cellular genome is a highly critical event during which cells are extremely susceptible to agents that disturbs the tightly regulated synthesis and utilization of DNA precursors.

The enzyme has been reported to have antitumour activity by Kusakabe *et al.* in the year 1979 and the enzyme has been partially characterized. Later on detailed characterization and exploration of the enzyme for its antitumour property was not observed. The reasons are unclear at present and this tempted us to speculate and investigate the biological properties of pterin deaminase. Thus the study lay a foundation and fling light for further exploration for cancer therapy.

In the present study, the biological implications of pterin deaminase from *Micrococcus luteus* BI252 and rat liver were studied on human lymphocyte culture, two human breast cancer cell

lines MCF-7 and MDAB231, and on HEp-2 cell lines. Cell death induced by apoptosis was observed in MCF-7 cell lines where as no such effect was observed in MDAB231 cell lines. At 350µg/mL enzyme concentration 50% inhibition was observed in MCF-7 cell lines. Cytopathic effect of pterin deaminase was observed on HEp-2 cell lines.

### **Pterin deaminase - Unexplored Enzyme from Rat Liver Exhibiting Antitumour Activity**

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Antifolates a class of antimetabolite drugs widely used in cancer therapy inhibit folate dependent enzymes and exert a powerful impact on folate dependent cell division resulting in inhibition of proliferation leading to cell death.

Asparaginase is an anti-neoplastic agent, used in lymphoblastic leukemia chemotherapy. Similarly, the most unexplored and possibly a potent drug is the enzyme pterin deaminase (E.C. 3.5.4.11), which deaminates folic acid to products 2-hydroxy-2-deamino pteroyl glutamic acid and ammonia. The deaminated product could not be utilized for the DNA synthesis as it differs structurally which results in folate deficiency, thus reduces the proliferation of cancerous cells. In January 1976, U.S. Patent No. 3,930,955 was awarded to Kusakabe *et al.* for the process of producing pterin deaminase having antitumour activity. This enzyme holds much promise in cancer therapy because its action is independent of the level of DHFR. This enzyme was able to inhibit the growth of L5117Y mouse leukemic cells *in vitro* and Walker 256 carcinoma *in vivo*.

In our study, pterin deaminase enzyme was isolated from rat liver with enzyme activity of 9.08 IU/mg. The enzyme was sequentially purified

using Ion-exchange and G-75 Gel Filtration column chromatography which yielded an enzyme activity of 14.796 IU/mg and 52.272 IU/mg respectively. The purification of pterin deaminase from rat liver was obtained as 6-fold purity with respect to the crude. The purified enzyme was characterized for its optimum pH, optimum temperature, temperature stability, kinetic properties and the inhibitors effect on pterin deaminase.

In spite of the patent and exploration of the preliminary antitumour property of enzyme pterin deaminase and its various roles in prokaryotes and eukaryotes, very few works has been carried out in elucidating the properties of the enzyme. The reasons behind the lack of work are unclear currently and this tempted us to speculate and investigate the properties of pterin deaminase from rat liver. Since the enzyme is present in rat liver, we believe that the enzyme would be there in human with an unknown function. Thus the present work renovate the basic study of characterizing the enzyme pterin deaminase which would lay a foundation and fling light for further exploration for cancer therapy.

### **Phenylalanine and Tyrosine Measurements Using HPLC with either UV or Fluorescence Detection**

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Phenylalanine (4)-hydroxylase converts phenylalanine (Phe) to tyrosine (Tyr) concentrations and represents the first step in the biosynthesis of important neurotransmitters like DOPA, dopamine, adrenaline and noradrenaline. An altered Phe to Tyr ratio (Phe/tyr) is indicative for a disturbed hydroxylation reaction which can be detected by parallel measurements of Phe and Tyr (1). Concentrations of the two amino acids can be determined with different laboratory methods, whereby ion exchange chromatography (HPLC) with ninhydrin derivatisation is up to now the

most frequently used (2). However, these methods usually are devoted to measure several amino acids and are therefore quite time consuming, analysis time of one single run often exceeding 60 min. Recently a new HPLC-method on reversed phase using specific fluorescence detection was developed, which allows measurement of one specimen within less than 7 minutes (3, 4).

In this study, we compared both these methods investigating 20 random plasma samples including patients with overt phenylalanine (4)-hydroxylase abnormalities. Phe and Tyr concentrations were determined using ion exchange chromatography with ninhydrin detection (method 1; Ref. 2) and HPLC with fluorescence detection at 210 nm excitation and 302 nm emission wavelengths (method 2; Ref 3, 4). Average Phe concentrations were lower with method 1 (128  $\mu\text{mol/L}$ ) as compared with method 2 (141  $\mu\text{mol/L}$  = 110% of method 1), whereas average Tyr concentrations were slightly higher with method 1 (56.0  $\mu\text{mol/L}$ ) as compared with method 2 (54.8  $\mu\text{mol/L}$  = 97.8% of method 1). Strong correlations existed between the concentrations measured by the two methods (Phe:  $R_2 = 0.996$ , Tyr:  $R_2 = 0.968$ , Phe/Tyr:  $R_2 = 0.996$ , all  $p < 0.0001$ )

Results show that both methods gave comparable results for the determination of Phe and Tyr concentrations in human serum specimens, albeit with a significantly shorter run time with the fluorescence detection method after reversed phase chromatography.

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#### **Influence of Immunosuppressive Agents on Neopterin Production and Tryptophan Degradation in Human Peripheral Blood Mononuclear Cells**

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Several potent immunosuppressive drugs are currently available to prevent acute rejection following solid organ transplantation. However, most of these agents are quite unspecific and exhibit serious side effects. There is a current need to search for more defined approaches to inhibit the alloimmune response such as the use of monoclonal antibodies. In experimental models novel agents like the costimulatory blocker CTLA4-Ig have been shown to inhibit T-cell activation and to induce donor antigen-specific tolerance. Recently, tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) has been demonstrated as a crucial mediator of CTLA4-Ig-induced tolerance. Like IDO also neopterin production is induced in human monocyte-derived macrophages and dendritic cells upon stimulation with Th1-type cytokine interferon- $\gamma$ . However, a possible interaction between IDO activity and conventional immunosuppressive agents has not been investigated yet. This study examined the influence of conventional immunosuppressants tacrolimus (FK506, Prograf), cyclosporin A (CsA, Sandimmune), mycophenolate-mofetil (MMF, CellCept), sirolimus (Rapamune), prednisolon and methylprednisolon (Urbason) on stimulated and unstimulated freshly isolated human peripheral blood mononuclear cells (PBMC) from healthy donors.  $10^6$  cells/ml unstimulated PBMC were exposed to increasing concentrations (0.005 - 10  $\mu\text{g/ml}$ ) of compounds and then after 30 min. either stimulated or not with 10  $\mu\text{g/ml}$  phytohaemagglutinin (PHA). After 48 hours of incubation, neopterin, tryptophan and kynurenine concentrations in cell culture supernatants were analyzed by high performance liquid chromatography (HPLC). Kynurenine to tryptophan ratios (kyn/trp) were calculated as an indirect estimate of functional IDO activity.

In PBMC, PHA significantly induced neopterin production and tryptophan degradation in a dose-dependent manner. With the exception of MMF, all compounds significantly diminished sponta-

neous neopterin production and tryptophan degradation in PBMC at the lowest concentrations applied, however sirolimus inhibited kyn/trp only at a concentration >1 µg/ml. PHA-induced neopterin production and tryptophan degradation was significantly inhibited by FK506, CsA and sirolimus at >0.1 µg/ml concentration, for MMF, prednisolon and methylprednisolon concentration higher than 10 µg/ml was required to achieve a significant reduction of kyn/trp. These latter compounds had higher capacity to inhibit neopterin production than tryptophan degradation.

Results of this study provide further evidence that the effects of various immunosuppressants like CsA, FK506 and sirolimus on IDO activity may differ which may relate to their capacity to either inhibit, or synergize with tolerance induction, when used in combination with T-cell costimulatory blocking agents.

### **Tim-3 Blockade Aggravates Anti-GBM Glomerulonephritis in a Th2 Mouse Model**

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T cell immunoglobulin and mucin protein (Tim-3) attracts attention as important cell surface protein that negatively regulates Th1-type responses. Blockade of Tim-3 has been shown to aggravate Th1-dependent disease models, such as experimental allergic encephalomyelitis and autoimmune induced diabetes mellitus type I.

To elucidate the role of Tim-3 in the TH-1 dependent autologous phase of anti-glomerular basement membrane (GBM) glomerulonephritis (GN), we first evaluated Tim-3 expression in mice after induction of anti-GBM GN. Furthermore, mice were treated with a Tim-3 blocking monoclonal antibody or an isotype control, anti-GBM GN was induced and mice were followed for 7 days.

Significantly increased Tim-3 expression was

found in kidneys, but not in lymph nodes, 4 and 8 weeks after induction of anti-GBM GN. Tim-3 expression correlated with the expression of the master switch gene of Th1-type cells t-bet. Administration of anti-Tim-3 antibody aggravated anti-GBM GN as shown by significantly increased albuminuria, histological changes, lipocalin-2 and IL-18 levels. Significantly increased numbers of macrophages were detected to infiltrate kidneys of mice after anti-Tim3 antibody treatment. T cell infiltration and Th1-type cytokine expression in kidneys was marginally affected by Tim-3 blockade. In contrast, no difference in immune regulation was found in secondary lymphoid organs.

Thus, Tim-3 is a key inhibitory signal on differentiated Th1-type cells infiltrating kidneys after induction of anti-GBM GN thereby limiting local macrophage infiltration and/or proliferation.

### **Everyday Dynamics of IL-6 and Neopterin Levels in a Breast Cancer Patient with Cancer-related Fatigue**

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While neopterin has clearly been shown to be a pro-inflammatory marker of immune activity in cancer, interleukin-6 (IL-6) lacks consistent evidence regarding either Th1 or Th2 affiliation. In this integrative single-case study on a 49-year-old female patient with breast cancer (diagnosis 5 years ago, pT2, pN1biv, cM0, G3, HER2+/neu+) and cancer-related fatigue we studied the functional dynamics of IL-6 and neopterin levels under real-life conditions. For this purpose, the patient collected her whole urine over a period of 27 days, divided into 12-hour portions. Moreover, she filled in questionnaires on emotional state,

daily routine, subjective illness activity and daily occurrences. Weekly, the patient was clinically checked and interviewed in order to get information on the past week's emotionally negative and positive everyday incidents. Urinary neopterin was measured by HPLC, urinary IL-6 by ELISA. Intensity and emotional valence of incidents were rated via consensus rating. Time series analysis consisted of ARMA modelling and cross-correlational analysis. This study showed sharp contrasting biphasic patterns in neopterin and IL-6 irrespective whether negative or positive incidents were used as triggers of the stress system. Preliminary analysis revealed that negative incidents were initially followed by increases in IL-6 within 12 h after the occurrence of stressors (lag0: +0.228; n.s.) and then by decreases after a total of 132 h (lag11: -0.326;  $p < 0.05$ ). Positive incidents, moreover, were followed first by decreases in IL-6 after 12 h (lag1: -0.332;  $p < 0.05$ ) and then by increases a total of 144h later (lag12: +0.303;  $p < 0.05$ ). Neopterin, in turn, first decreased 12 h after the occurrence of stressors (lag1: -0.352;  $p < 0.05$ ) and then increased after a total of 96 h (lag8: +0.308;  $p < 0.05$ ). In response to positive incidents, however, neopterin first increased after 36 h (lag3: +0.289;  $p < 0.05$ ) and then decreased after a total of 84 h (lag7: -0.255;  $p < 0.05$ ). This study showed for the first time sharply contrasting dynamic response patterns of neopterin and IL-6 under real-life conditions suggesting that IL-6 could be an indicator of anti-inflammatory marker of immune activity in breast cancer. Further integrative single-case studies have to be done in order to generalize findings on this topic.

### **Impact of Iron Treatment on the Immune Effector Function of Macrophages in Dialysed Patients**

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Anemia in dialysis patients is multifactorial

and can be partly referred to erythropoietin deficiency which is often associated with an impaired iron availability for erythropoiesis due to chronic inflammation. Thus, anemia is treated with recombinant human erythropoietin (rhEPO) often in combination with iron. However, iron therapy may harbour some detrimental effects as it can serve as a nutrient for invading pathogens, modulate innate immune effector pathways of macrophages and promote intravascular radical formation.

We investigated a total of 24 patients on chronic hemodialysis who were withheld from treatment with iron for two weeks before study entry. The study was approved by the local ethical committee and written informed consent was obtained from all patients. While the intervention group ( $n = 16$ ) received a single parenteral dose of 100 mg iron gluconate at day 0, control patients ( $n = 8$ ) received the same volume of saline. Blood was drawn before iron injection at day 0 and after 48 hours and one week, immediately before dialysis. Monocytes were isolated for investigation of immune response and cellular iron homeostasis *ex vivo*.

Following iron administration we noticed a significant increase of iron, transferrin saturation and ferritin concentration in serum after 48 hours while hepcidin concentrations decreased. In parallel, iron application resulted in increased intracellular ferritin concentrations and a reduced transferrin receptor mRNA expression within blood monocytes. When monocytes were stimulated *ex vivo* with LPS or interferon-gamma we found that high circulating ferritin concentrations at study entry were associated with a decreased expression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). In contrast, 48 hours after iron application we observed a transient increase of TNF- $\alpha$  and IL-6 formation in monocytes of patients with low circulating ferritin levels at study entry as compared to monocytes from dialysis patients not receiving iron therapy.

Iron therapy in chronic dialysis patients affects the immune function of monocytes/macrophages. Prolonged iron therapy with systemic iron overload as reflected by increased circulating ferritin levels is associated with impaired secretion of

macrophage derived cytokines like IL-6 and TNF- $\alpha$  which may cause an increased susceptibility to infections. Thus, accurate monitoring of body iron status as well as new diagnostic tools to clearly assess iron availability in the circulation are urgently needed.

### **Compounds Found in Exhaled Breath of Patients Suffering from Lung Cancer**

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Analysis of exhaled breath with respect to concentrations of volatile organic compounds bears fascinating possibilities for medical diagnosis and therapeutic monitoring. Here we make an attempt to determine VOCs in exhaled breath of lung cancer patients. We restrict ourselves to compounds which show at least 15% higher concentrations in exhaled breath as compared to inhaled air.

The exhaled breath and inhaled room air samples from 65 lung cancer patients in different disease stages and different treatment regimes and 31 healthy controls have been analysed. Expiratory and indoor air samples were collected. Gas chromatography with mass spectrometric detection (GC-MS) and proton transfer reaction mass spectrometry (PTR-MS) were used.

Altogether 103 compounds showing at least 15% higher concentration in exhaled breath than in inhaled air were detected. All results reported here refer to concentrations being 15% higher in exhaled breath as compared with inhaled air. Among the 103 compounds detected, 84 were

confirmed by determination of the GC-MS retention time using pure compounds. Around one third of the compounds detected were hydrocarbons. We found hydrocarbons, alcohols, aldehydes, ketones, esters, ethers, sulfur compounds, nitrogen-containing compounds and halogenated compounds. Acetonitrile and benzene were among 11 compounds which correlated with smoking behaviour.

As a particular example for age and gender effects, we investigated the concentration of isoprene in 205 adult volunteers by PTR-MS: there was no statistically significant difference between mean isoprene levels in the breath of males and females (GM 105.4 and 95.5 ppb, correspondingly). Aging caused decrease of the concentrations of the investigated VOC in men with an estimated slope of the regression line for the log-transformed isoprene concentrations of -0.0049, but did not influence isoprene level in women. PTR-MS analysis is very easy to be performed and therefore was used for studies including many volunteers.

A comparison of the results of cancer patients with those of 31 healthy volunteers revealed differences in the concentration patterns. Sensitivity for detection of lung cancer patients depends on the number of compounds used. Based on 8 different compounds (or 12 or 54 different compounds, respectively) not arising in exhaled breath of healthy volunteers the sensitivity was 51% (or 78% or 86%, respectively), the specificity always being 100%. Potential marker compounds are 1-propanol, 2-butanone, 3-butyn-2-ol, benzaldehyde, 2-methyl-pentane, 3-methyl-pentane, n-pentane and n-hexane.

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### **The Release and Uptake of Volatile Organic Compounds (VOCs) from Different Lung Cancer Cell Lines *in vitro***

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Analysis of exhaled breath for the detection of human diseases like lung cancer offers the possibility of non-invasive diagnosis. Little is known about the underlying metabolic mechanisms leading to the production of volatile organic compounds (VOCs). Furthermore, tumours are complex and heterogenous systems, and tumorous and non-tumorous components like immune cells and possibly also microorganisms could contribute to the spectrum of VOCs detected. The aim of this work was to confirm the existence of tumor-derived VOCs by analyzing various lung cancer cell lines

So far, four lung cell lines have been investigated for the release or consumption of VOCs by GC-MS after thermal desorption. Cells were trypsinized and up to  $100 \times 10^6$  cells were incubated in a sealed fermenter. Prior to gas chromatography mass spectrometry (GC-MS) analyses samples from the headspace of the culture vessel were collected with simultaneous preconcentration by adsorption on solid sorbents and then VOCs were thermodesorbed for analysis by GC-MS.

The results showed a clear increase compared to medium only controls in the concentrations of different branched hydrocarbons and partly also of alcohols and ketones in the case of NCI-H2087, CALU-1 and A549 cells. However, the patterns differed among these cell lines, although chemically similar branched hydrocarbons, for instance 2,3,4-trimethylpentane and 2,3,3-trimethylpentane, were found to be released.

Moreover, in all tested lung cancer cell lines tested, and also in NCI-H1666 cells, decreased concentrations of VOCs compared to controls could be demonstrated. Especially different aldehydes and the ester n-butyl acetate were consumed in all cell lines. Amongst the aldehydes, 3-methylbutanal was decreased in all cell lines, methacrolein in all cell lines except NCI-H2087 cells, 2-methylpropanal in all cell lines except NCI-H1666 and hexanal in all tested cell lines but not in A549 cells. For additional VOCs that decreased in concentration the compounds were restricted to the individual investigated cell line.

In summary, our findings confirm that several VOCs like unsaturated hydrocarbons and oxygen-containing compounds are released, while different aldehydes and the ester n-butyl acetate are consumed or metabolized by the cell lines studied here.

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### **Novel Antioxidative Effects of Folic Acid and Betaine in Humans.**

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Experimental evidence indicates that the homocysteine-induced damaging effects on various cell function and structures are mediated, in part, by increased generation of reactive oxygen species (ROS). Indeed, *in vitro*, most effects of homocysteine can be prevented by preincubation with antioxidants. Homocysteine -lowering treatment with folic acid and betaine (trimethyl-

glycine) has been effective in reducing cardiovascular in patients with homocystinuria and also in adults in absence of hyperhomocysteinemia. Thus, we investigated the potential pro-oxidant effects of folic acid and betaine *in vivo* in three independent investigations (A-C).

A: 27 male CAD-patients (46-56 years) were treated in a cross-over trial with folic acid (5mg/d) for 6 weeks. B: 67 healthy volunteers (mean 52 years) were randomized in 5 groups receiving placebo, 0.4mg, 1.2mg, 2.5mg or 5mg of folic acid/d for 8 weeks. C: 24 healthy male subjects (54 years) were randomized to receive either 2.5mg folic acid or 3g betaine per day for 2 weeks. We measured peroxides, peroxidase-activity, total antioxidant capacity (TAC), antibodies (IgM) against malondialdehyde (MDA) and against oxidized LDL-cholesterol (oLAB), Protein-Ox, Lipid-Ox in serum at various time points.

The main results can be summarized that FA (5.0 mg) significantly added TAC to serum and increased NO bioavailability (after 4 weeks) in healthy subjects and also in CAD-patients. FA (0.4 - 5.0mg) significantly protects lipids and proteins from oxidative modification as early as after 2 hours (as measured with lipidox and proteinox tests). Betaine intake had a rapid Hcy-lowering effect (-8.2% within 2h) and the baseline concentration (40%) increased nearly 17-fold. Betaine significantly increased peroxidase-activity (48% after 4h) and lowered peroxide levels (-18% after 6h; both  $p < 0.05$ ).

Our findings suggest antioxidative effects of folic acid and betaine, complimentary to homocysteine-lowering. These effects were observed *in vivo* and may explain beneficial treatment effects on the cardiovascular system observed in various clinical conditions.

### **Structural Insights into the Evolutionary History of Phenylalanine Hydroxylase**

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Phenylalanine hydroxylase (PAH) is a tetrame-

ric enzyme which in mammals is mainly present in the liver where it converts phenylalanine to tyrosine. In order to transform phenylalanine into tyrosine the enzyme is dependent on tetrahydrobiopterin as electron donor in the catalyzed reaction. Proper regulation of the mammalian enzyme is very important to maintain the appropriate level of phenylalanine to (i) avoid the disease phenylketonuria (PKU) characterised by mental retardation and (ii) provide a continuous supply of tyrosine for protein synthesis. One important regulatory mechanism is the cooperative response to phenylalanine binding in the active sites of the four subunits. The biopterin cofactor has an inhibitory effect on PAH activity and at the same time protects the enzyme from degradation.

We have compared PAH isolated from the nematode *Caenorhabditis elegans* (cePAH) with the human form of the enzyme (hPAH). Work done in our lab suggests that the main function of cePAH is anabolic using phenylalanine as a precursor of melanin. Although tetrameric in structure it lacks the sophisticated regulatory mechanisms found in the mammalian enzyme. This implies that the importance of proper regulation and prompt response of the enzyme to elevated substrate level have evolved with the complexity of the nervous system and the need for protection against neurotoxic phenylalanine levels.

We have constructed structural models of the full length hPAH and cePAH based on crystal structures of truncated forms and homology modelling. Molecular dynamics simulations have been applied to these models to get insight into the conformational changes related to regulation by both the substrate and biopterin cofactor at an atomic level.

This work has identified several amino acid residues important for regulating the mammalian enzyme. It has also provided insights into the basis for the evolution of sophisticated regulatory mechanisms in an important metabolic enzyme.

### **Diagnostic Procedures and Treatment in Renal Transplant Patients with Polyoma (BK) Virus Infection**

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After the first description of a new papovavirus by Gardener (1971), it took nearly 25 years to learn about the impact of modern immunosuppressive therapy on the outcome of polyoma virus infection after renal transplantation. About 80 % of the general population have detectable antibodies to BK virus, the prevalence of BK viremia, viremia and nephritis is 30, 13, and 8%, respectively. Diagnosis in renal transplant patients can be made by finding Decoy-cells in urine, or by polyoma virus specific PCR in urine or blood. We report on diagnostic procedures for BK virus infection in our transplant patients and the follow-up results of BK infected patients in our clinic since 2001. Treatment of BK virus nephropathy was performed by reducing the immunosuppressive therapy and guided by intensive monitoring of infectious relapse or rejection episodes. When graft function was impaired by inflammation, we observed no progression after viral clearance. No patient with BK virus nephropathy lost his graft. However, we learnt that for efficient disease management an early diagnosis, robust differentiation between viral infection and rejection and avoidance of excessive immunosuppression are key. In this respect non-invasive markers such as neopterin might be of great diagnostic importance.

### **Regulation of Iron Homeostasis in Anemia of Chronic Disease and Iron Deficiency Anemia. Diagnostic and Therapeutic Implications**

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Anemia of chronic disease (ACD), also termed anemia of inflammation (AI), is characterized by macrophage iron retention induced by cytokines and the master regulator hepcidin, which controls cellular iron efflux upon binding to the iron export protein ferroportin. Many patients, however, present with a combination of ACD and true iron deficiency (ACD/IDA) secondary to chronic bleeding, which is challenging to diagnose.

To study the different underlying regulatory pathways, we used a rat model of chronic arthritis developing persistent ACD, and mimicked ACD/IDA by phlebotomies. Iron retention during inflammation occurred in macrophages and the spleen, but not in the liver. In rats and humans suffering from ACD, serum hepcidin concentrations were elevated, which was paralleled by reduced duodenal and macrophage expression of ferroportin. Importantly, individuals suffering from ACD/IDA had significantly lower hepcidin levels than ACD subjects, and ACD/IDA individuals in contrast to ACD subjects were able to absorb dietary iron from the gut and to mobilize iron from macrophages.

Circulating hepcidin levels affect iron traffic in ACD and ACD/IDA and are rather regulated by the erythropoietic needs for iron than by inflammation. Hepcidin determination can differentiate between ACD and ACD/IDA, which will help clinicians to select the appropriate therapy for these patients.

### Properties of Glyceryl ether monooxygenase

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Besides the four well characterised tetrahydrobiopterin-dependent enzymes, the nitric oxide synthases and the aromatic amino acid hydroxylases, there still remains a fifth but less well described enzyme, the glyceryl ether monooxygenase (alkyl glycerol monooxygenase, EC 1.16.14.5). Although increasing information about this protein is accessible, no sequence has been identified and therefore it belongs to the category of orphan enzymes. It has been shown that this enzyme critically depends on tetrahydrobiopterin as cofactor for intact catalytic activity while common reductants are not able to substitute for the pterin cofactor and needs a non-heme bound metal ion for its catalytic function. Therefore, glyceryl ether monooxygenase is more closely related to the family of aromatic amino acid hydroxylases (using a non-heme iron) than to the nitric oxide synthases which fulfil their catalytic activity via a heme iron. In addition, it has been shown that this enzyme is a membrane-bound protein with highest activity levels detected in male rat livers.

In order to increase the understanding about the physiological role of glyceryl ether monooxygenase and possible regulators we investigated the expression levels in various mice tissues. Measurable amounts of glyceryl ether monooxygenase activity (higher than 1 pmol/(mg\*min)) were detected in subcutaneous and visceral fat tissue, stomach, testis, adrenal gland, lung and kidney. Less than 1 pmol/(mg\*min) was measured in

spleen, intestine, brain, skeletal muscle, ovary and heart. This expression pattern resembles the results obtained in rats with a single exception, the liver, where expression was strongly decreased while rats always displayed very high levels of glyceryl ether monooxygenase in this organ (18.0 pmol/(mg\*min) in mice versus 1253.5 pmol/(mg\*min) in rats).

These results now encourage us to analyse glyceryl ether monooxygenase activity in livers from mice harbouring engineered genetic defects in general lipid regulators like the peroxisome proliferator-activated receptor  $\alpha$ .

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### Motifs for Tetrahydrobiopterin Binding in Mammalian Enzymes

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Of the five tetrahydrobiopterin dependent enzyme reactions known in mammals, four have clearly defined sequences and genes, and structures are known. These are the aromatic amino acid hydroxylases phenylalanine hydroxylase (gene symbol PAH), tyrosine hydroxylase (gene symbol TH), and the two tryptophan hydroxylases (THP1 and TPH2). Another group is formed by the nitric oxide synthases (gene symbols NOS1, NOS2 and NOS3 for the neuronal, macrophage and endothelial isoform, respectively). The fifth is alkylglycerol monooxygenase (glyceryl ether monooxygenase), an enzyme with unknown sequence. To enable a search for candidate sequences for this enzyme in protein databases, we compared features that are required for tetrahydrobiopterin binding and utilisation in aromatic amino acid hydroxylases and nitric oxide synthases.

Aromatic amino acid hydroxylases bind tetrahydrobiopterin with 14 amino acid residues, 7 of which are conserved. They are distributed

over a region of approximately 80 amino acids, and come all from the same peptide chain. Nitric oxide synthase oxygenase domains, in contrast, use residues from two different peptide chains of the dimer to bind tetrahydrobiopterin. Ten residues are close to tetrahydrobiopterin, 5 of which are conserved. They are distributed over a region of approximately 360 amino acids. Together with the lacking amino acid sequence homology between aromatic amino acid hydroxylases and nitric oxide synthases, and the fundamentally different biochemistry of cofactor usage by these two enzyme classes, this suggests that aromatic amino acid hydroxylases and nitric oxide synthases evolved as separate classes of tetrahydrobiopterin utilising enzymes.

Alkyl glycerol monooxygenase resembles the aromatic amino acid hydroxylases rather than the nitric oxide synthases with respect to handling of the tetrahydrobiopterin cofactor, while the chemistry of the substrate hydroxylation is different to both, aromatic amino acid hydroxylases and nitric oxide synthases. In alkyl glycerol monooxygenase, a saturated aliphatic carbon is hydroxylated, whereas the aromatic amino acid hydroxylases hydroxylate a carbon of an aromatic ring, and nitric oxide synthases hydroxylate a nitrogen of the guanidino group of arginine. In the mammalian proteomes, no significant primary sequence homologue to aromatic amino acid hydroxylases can be found by various methods. To look for alkyl glycerol monooxygenase candidate genes, we therefore plan to search for proteins with structural similarities to aromatic amino acid hydroxylases, hoping that these structural similarities may reflect the similarity in tetrahydrobiopterin cofactor utilisation.

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### **Circulating NT-proCNP in Patients after (multiple) Trauma or Burns**

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Atrial, brain and C-type natriuretic peptides (ANP, BNP and CNP, respectively) are the known members of the mammalian natriuretic peptide system. The biological properties of CNP differ notably from those of ANP and BNP. In contrast to these cardiac peptides, CNP is primarily expressed in non-cardiac tissues like the central nervous system or the vascular endothelium exhibiting various local paracrine or autocrine functions and thus usually not being considered an endocrine hormone. Indeed, the function of CNP, first isolated from porcine brain, is still being elucidated. In humans the transcription of the gene is regulated by factors such as TNF- $\alpha$ . All natriuretic peptides are produced as propeptides, which are subsequently cleaved into the biologically active C-terminal hormone and the N-terminal fragment. Since those N-terminal fragments are much more stable, and circulate in higher amounts than the active peptides, they are easier and more reliably detectable in serum or plasma. We examined the pathophysiological significance of plasma NT-proCNP levels following different qualities of traumatic injury with relationship to injury severity and outcome employing a sandwich immunoassay using antibodies directed against amino acids 1-19 and 30-50 (Biomedica, Vienna, Austria). In this study 4 cohorts were included comprising 30 patients suffering from severe multiple injuries (T), 31 burns (B), 41 patients who underwent elective abdominal surgery, and 30 healthy volunteers. Systemic NT-proCNP levels were assayed on admission (accidental trauma) or pre-op (elective surgery), respectively, and on consecutive days d1, d3, d5 and d7. Differences in the experimental means were considered to be significant if  $p < 0.05$ , as determined by ANOVA and adequate post hoc procedures for multiple comparisons, where appropriate. Injury severity was assessed with the Injury Severity Score (ISS) in T and % burned total body surface area (%TBSA) in B.

Plasma NT-proCNP was significantly diminished in burns on admission and day 1 postburn

compared to controls, but in the further course on days 3 and 5 higher than on day 1. Peak levels were detectable on day 5 postburn. Different from burns NT-proCNP levels remained on control level after severe multiple injury. In contrast, NT-proCNP gradually increased following elective surgery showing highest levels on day 7 compared to pre-op and day 1 post surgery. Moreover, NT-proCNP levels were significantly higher in patients developing septic complications after elective surgery. In burns no correlation was found with inhalation injury or percentage of burned body surface. But, it is noteworthy that NT-proCNP values were significantly lower in survivors compared to controls, whereas levels in

non-survivors remained unchanged. In polytraumatized patients, however, NT-proCNP levels increased with injury severity. Finally, in burns a preliminary correlation analysis revealed significant relationships of NT-proCNP to neopterin ( $r_s = 0.452$ ,  $p < 0.001$ ), C-reactive protein ( $r_s = 0.334$ ;  $p < 0.001$ ) and TNF- $\alpha$  ( $r_s = 0.543$ ;  $p < 0.001$ ), but to a lesser extent with the development of organ dysfunction (SOFA). Even if NT-proCNP levels are obviously associated with markers of immune activation and inflammation, their posttraumatic implications and especially their prognostic value for the development of septic complications remain questionable and require further investigations.