Pteridines

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**Monitoring of immune activation by biochemical
changes in porcine models**

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In animal models, the assessment of immune activation is often difficult because of the limited availability of specific assays to detect cytokine activities. In human monocytes/macrophages, interferon- γ induces increased production of neopterin and an enhanced activity of indoleamine 2,3-dioxygenase (IDO) which degrades tryptophan via the kynurenine pathway. Therefore, the monitoring of neopterin concentrations and of tryptophan degradation can be used to detect the extent of Th1-type immune activation during cellular immune response in humans. In a porcine model of cardiac arrest we re-examined the potential use of neopterin measurements and the determination of the tryptophan degradation rate, respectively, for the estimation of the extent of immune activation. In brief, a saline-filled catheter was advanced into the abdominal aorta for measurement of aortic blood pressure and gaining blood samples. Fifteen minutes before cardiac arrest, 5000 U heparin were administered intravenously to prevent intracardiac clot formation. Instead of external manual chest compression, a heart lung machine was used to control the blood flow in this reanimation model. Three episodes of cardiac arrest were induced by a 50-Hz, 60-V alternating current applied via two subcutaneous needle electrodes, and ventilation was stopped at that point. The total duration of the episodes was 22 min (episode 1) and 10-14 min (episodes 2 and 3). The total duration of the experiment was 5 hours. Blood samples were collected from a catheter in the arteria

femoralis immediately after instrumentation to give the baseline values. Further specimens were drawn each before start of the first episode and the second episode as well as at the end of the experiment before necropsy.

Urinary neopterin concentrations were measured by HPLC and radioimmunoassay (BRAHMS Diagnostica, Berlin, Germany). Serum and plasma tryptophan and kynurenine concentrations were determined by HPLC. Serum and urine neopterin concentrations were not detectable in these specimens. The mean serum tryptophan concentration was 39.0 ± 6.2 $\mu\text{mol/l}$, and the mean kynurenine concentration was 0.85 ± 0.33 $\mu\text{mol/l}$. The average kynurenine per tryptophan quotient in serum was 21.7 ± 8.4 $\mu\text{mol/mol}$ and in plasma 20.7 ± 9.5 ($n=7$).

We gained preliminary data for a potential use of monitoring tryptophan degradation but not neopterin concentrations to detect immune activation in a porcine model. The kynurenine per tryptophan quotient may serve for short-term measurement of immune activation and hence enables an estimate for the extent of immune activation.

Surface heparinization reduces complement activation during cardiopulmonary bypass

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Cardiac surgery induces a systemic inflammatory response. The mechanisms are not fully understood but surgical trauma, cardiopulmonary bypass (CPB), cardiomyotomy suction, heparin/protamine complex, myocardial ischemia and autotransfusion have been suggested

to contribute. The inflammatory response can be detected in blood by an increase in cytokine and complement concentrations during and early after surgery. During CPB, the contact between blood and non-heparinized surfaces, closed circuits and pump type have been suggested to be of importance. The combined effect of surface heparinization on complement activation during and after CPB was studied. Forty-one adult patients undergoing coronary bypass surgery (CABG) and/or aortic valve replacement (AVR) with an expected CPB time of more than 90 minutes participated in a prospective, controlled and randomised study. The patients were allocated into two groups: a treatment group (Group A, n=21) were the extracorporeal system consisted of a Biomedicus centrifugal pump and a closed heparinized circuit (Carmeda Bioactive Surface, CBAS), and a control group (Group B, n=20) were the extracorporeal circuit consisted of a conventional, nonheparinized system with hard shell venous reservoir and roller pump. There were no differences between the groups with respect to age, aortic clamping time, CPB time or type of operation. SC5b-9 increased during and early after CPB in both groups. The increase was significantly more pronounced in the conventional group. There were no differences regarding the release of neopterin. The combined use of surface heparinization reduces complement activation substantially during and early after cardiopulmonary bypass.

Evidence for inflammatory cyclooxygenase-2 expression and prostaglandin formation in human thyroid epithelial cells

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To investigate Cyclooxygenase isoenzyme expression in the thyroid gland in auto-immune thyroid diseases and in the thyroid epithelial cell line Nthy-ori 3-1, COX-1/-2 proteins were analysed by immunohistochemistry in thyroid tissue sections from Hashimoto's thyroiditis, Graves' disease and non-inflamed thyroids. In vitro COX-1/-2 gene expression was examined in the thyroid epithelial cell line (Nthy-ori 3-1) using semi-quantitative RT-PCR and Western blot analysis. ELISAs were employed to assess whole cell COX-enzyme activity, PGE₂ and IL-6 formation. Immuno-

reactive staining of COX-2 protein was marked in thyroid epithelial cells of tissue sections from Hashimoto's thyroiditis and from Graves' disease, where as in non-inflamed thyroid tissues immunoreactive COX-2 staining was only moderate. COX-1 protein was only scarcely detectable in all thyroid tissues analysed. In vitro, IL-1 β and TNF- α combined elicited a marked time-dependent induction of PGE₂ release in the thyroid epithelial cells. This was reflected by increased levels of COX-2 specific mRNA, increased amounts of COX-2 protein and COX enzyme activity. The inhibition of induced COX enzyme activity by the COX-2 selective inhibitor, NS-398, demonstrated the presence of COX-2 pharmacologically. The induced PGE₂ release was also paralleled by a marked induction of IL-6 formation.

We provide evidence that COX-2 is induced in thyroid epithelial cells in Hashimoto's thyroiditis and in Graves' disease suggestive of increased PG formation in the inflamed thyroid gland. Our in vitro observations present first evidence that IL-1 β and TNF- α induce COX-2 gene expression and prostaglandin synthesis in thyroid epithelial cells. The discovery of COX-2 gene expression in the thyroid gland of autoimmune thyroid diseases raises the question whether a COX or COX-2 inhibitory treatment may elicit beneficiary anti-inflammatory effects to the patient with these diseases.

Pteridines and the brain

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Our early knowledge of the chemistry of the brain is chronicled in the Proceedings of the Royal Society of London. One of its founders, Robert Boyle, laid the foundations of biochemistry in his book "The Sceptical Chymist" (1661) and Thudicum, another Fellow of the Society, wrote the first "Chemistry of the Brain" (1885). A decade later in the Proceedings of the Royal Society Gowland Hopkins reported his findings of pteridines in the Brimstone Yellow butterfly (genus *pteridaceae*) (1895). This led him on a voyage of discovery, which culminated in the award of the Nobel Prize for medicine in 1950 for his work on vitamins. The importance of folic acid as a coenzyme has led to important insights into its role in the development of the nervous system, for example in neuronal migration and neural cleft closure and this has led to the effective prevention of spina bifida by the use of folic acid supplements in pregnancy. The discovery of biopterin in

the African Green Mamba, by John Blair in 1953, while he was working at the University of the Gold Coast (Ghana), led to a lifetime quest for elucidation of the role of pteridines in disorders of brain development including particularly phenylketonuria, childhood autism and Down Syndrome and in neurodegenerative disorders such as Alzheimer's and Parkinson's Disease and other dementias. It is no coincidence that the Nobel Prize for medicine for the year 2000 was awarded for advances in the treatment of Alzheimer's and Parkinson's diseases and Birmingham has been at the forefront of these developments. John Blair's contribution was more in the tradition of Lewy and the produced hypotheses for the chemistry of these disorders, involving pteridines, metals and immunological mechanisms. With the mapping of the human genome and our greater understanding of the phenotypic heterogeneity of these clinical disorders the quest is to provide non-invasive treatments for diseases of the brain and mind which are the plagues of our modern society. John Blair died in September 2000.

Effect of neopterin and 7,8-dihydroneopterin on intracellular calcium in NT2 cells

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Activated cell-mediated immunity, associated e.g. with human immunodeficiency virus infection is accompanied by elevated concentrations of neopterin and 7,8-dihydroneopterin. Recent data reported an apoptosis-inducing effect of neopterin derivatives on various cell types, including Jurkat T lymphocytes, NT2 and hNT cells and PC12 cells - differentiated and non differentiated. Increase of $[Ca^{2+}]_i$ has been implicated as a mediator in apoptotic cell death. Earlier studies in THP-1 cells revealed that, 7,8-dihydroneopterin, neopterin and 5,6,7,8-tetrahydrobiopterin led to an increase of $[Ca^{2+}]_i$. Therefore we investigated whether calcium plays a role in pteridine-mediated apoptosis in human neuronal NT2 cells. Intracellular Ca^{2+} changes in NT2 cells were measured using the Ca^{2+} sensitive dye fluo-4 (Molecular Probes, OR, USA). $[Ca^{2+}]_i$ was measured in cells by imaging of fluo-4 at increasing time points following exposure to various concentrations of 7,8-dihydroneopterin,

neopterin and combinations. Upon incubation with 5 mM 7,8-dihydroneopterin and 1mM neopterin over 3 min we observed a peak in $[Ca^{2+}]_i$ after 40 sec, ceasing hereafter. The effect of 7,8-dihydroneopterin was concentration-dependent (50 to 5000 mM). Coincubation of 7,8-dihydroneopterin and neopterin in low concentrations did not superinduce the increase of $[Ca^{2+}]_i$. Krebs-Ringer buffer alone did not induce $[Ca^{2+}]_i$. As expected NT2 cells responded to the addition of ATP. In earlier findings, an involvement of reactive oxygen intermediates in pteridine-mediated apoptosis was shown in this cell line by coinubation with antioxidants. We found that the addition of the antioxidant, ammonia-pyrrolidinedithio-carbamate (PDTC), resulted in a significant decrease of the pteridine-mediated calcium signal, while PDTC alone did not evoke any calcium signal. Our findings in NT2 cells implicate that elevated and lower concentrations of 7,8-dihydroneopterin and neopterin increase $[Ca^{2+}]_i$ in NT2 cells and this might be involved as first signal in the cascade leading to redox-sensitive pteridine-mediated apoptosis.

Pteridine induced apoptosis in PC12 cells - involvement of MAPKinase and JNKinase

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In cerebrospinal fluid of patients with cerebral infections, elevated concentrations of the pteridine compounds neopterin and 7,8-dihydroneopterin were detected. Beside increased production of pteridines, proinflammatory cytokines such as tumour necrosis factor- α (TNF- α) and reactive oxygen intermediates were observed. Here, the potential of the pteridines to induce apoptosis of the rat pheochromocytoma cells (PC12) was investigated. Interference of neopterin and 7,8-dihydroneopterin with redox-sensitive intracellular signalling pathways has been found recently, thus we assessed potential role of the neopterin-derivatives in oxidative stress-mediated apoptosis. Further, involvement of mitogen activated protein kinase (MAPK) and stress activated protein kinase (SAPK or JNK) has been investigated in non-differentiated PC12 cells. PC12 cells, non differentiated and differentiated - present an interesting model for studying the impacts of the

pteridines on the loss of neurons in neurodegenerative diseases, since this cell line has the ability to differentiate into sympathetic neurons by exposure to nerve growth factor- β (NGF- β).

In contrast to aromatic pteridines like neopterin and biopterin, the reduced forms 7,8-dihydroneopterin (5 mM), 5,6,7,8-tetrahydrobiopterin (0.2 mM) and 7,8-dihydrobiopterin (1 mM) led to a significant increase of apoptotic cells. After terminal differentiation, cells were less sensitive to incubation with pteridines. A noticeable augmentation of apoptosis was observed upon incubation with 7,8-dihydroneopterin and 7,8-dihydrofolic acid. Antioxidants, ammonia-pyrrolidinedithio-carbamate (PDTTC, 0.1 mM), N-acetylcysteine (NAC, 5 mM), superoxidedismutase (SOD, 500 units/ml) and catalase (CAT, 500 units/ml), partly protected PC12 cells from pteridine induced-apoptosis, suggesting the involvement of reactive oxygen intermediates. The inhibiting effect of the antioxidants was most obvious in differentiated PC12 cells since the level of inhibition of spontaneous apoptosis in differentiated PC12 cells was very low. Investigation of the signalling cascades showed an involvement of the MAPK and JNK pathway. Activation of MAPK pathway leads to differentiation, proliferation or general survival signals, whereas SAPK activation generally results in stress responses, e.g. apoptosis or growth arrest). We observed an increase in activation of extracellular regulated kinase 1 and 2 (ERK1 and 2 - both belonging to the MAPK cascade) after incubation with 5 mM 7,8-dihydroneopterin within 5 min which ceased after 2 hours.

Our findings for the PC12 cells indicated that pteridines, especially reduced forms, potentially induce apoptosis in non-differentiated and NGF differentiated, sympathetic neuron-like PC12 cells. According to our observations pteridines lead to apoptosis via induction of reactive oxygen intermediates. Further, we could find an involvement of MAPK and JNK pathways in the 7,8-dihydroneopterin mediated signalling cascade, and activation could be altered by addition of TNF- α .

Iron and natural resistance: Regulatory interactions between iNOS expression, natural resistance associated macrophage protein 1 (NRAMP1) function and iron metabolism

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Iron homeostasis has profound effects on macrophage effector function and cellular immune regulation. While cytokines affect the expression of critical iron proteins and thus cause disturbances in iron homeostasis iron by itself modulates the activity of immune effector mechanism mainly by influencing IFN-gamma mediated effector pathways towards macrophages. Thus, macrophages loaded with iron lose their ability to kill intracellular pathogens via interferon-gamma mediated pathways. This can be in part referred to the inhibitory effect of the metal on cytokine inducible formation of NO by macrophages. This is due to a transcriptional mechanism by which iron impairs the binding of the transcription factor NF-IL6 to the inducible nitric oxide synthase (iNOS) promoter resulting in reduced iNOS transcription and NO formation. However, as Western blots indicate iron treatment does not affect NF-IL6 protein neither in cytoplasm nor within the nucleus. Instead, iron decreased NF-IL6 phosphorylation which is relevant for the transcription inducing force of the protein. Electromobility shift assays for NF-IL6 using inhibitors for various phosphorylation pathways now indicate that altered phosphorylation of NF-IL6 may be referred to a direct influence of the metal on phosphorylation processes exerted by a mitogen activated protein (map) kinase p-38 and tyrosine kinases. Moreover, we now provide new evidence that iron also affects the expression of natural resistance genes (NRAMP-1) which confers protection against infection with intracellular pathogens, such as Mycobacteria or Salmonella. Iron increased NRAMP-1 mRNA and protein expression which may be part of a negative feed back regulation by which increased intracellular iron perturbation increase the expression of the potential phagolysosomal iron transporter NRAMP-1. Even more interesting we also provide evidence that the functionality of NRAMP-1 influences the expression of iNOS which are both dependent on the availability of iron.

In summary, iron is able to modulate the expression of critical genes in host immune defense by a transcriptional mechanisms and influences the susceptibility and immune response mechanism towards intracellular infection by regulation NRAMP-1 function and iNOS expression.

Is hyperhomocysteinemia due to folic acid depletion rather than reduced dietary intake?

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Tetrahydrofolic acid is essential for the conversion of homocysteine to methionine thereby reducing the concentration of the former. Hyperhomocysteinemia is established as a risk factor for cardiovascular and cerebrovascular diseases. In subjects with hyperhomocysteinemia usually an inverse relationship exists to their folic acid levels, and supplementation with folic acid is able to lower homocysteine concentrations. Therefore, hyperhomocysteinemia is mainly ascribed to insufficient dietary intake of folic acid. Hyperhomocysteinemia has been found not only in patients with cardiovascular disorders but also in patients with dementia or disturbed glucose metabolism. Oxidative stress appears to be involved in the pathogenesis of these disorders, and associations have been found between homocysteine concentrations and the degree of immune system activation as it is indicated by, e.g., increased neopterin concentrations. Large amounts of neopterin are released by human monocytes/macrophages upon stimulation with interferon- γ , and neopterin concentrations were found to be associated with the production of reactive oxygen species. Thus, neopterin concentrations are indicative for cellular (=TH1-type) immune activation and allow to estimate the degree of oxidative stress elicited during immune response.

The fact that tetrahydrofolic acid is very susceptible to oxidation may become relevant under oxidative stress conditions. We assume immunologically-induced oxidative stress to be a main cause for endogenous folic acid depletion which results in hyperhomocysteinemia. Therefore, hyperhomocysteinemia is considered as an indirect consequence of hyperconsumption of antioxidant vitamins during prolonged states of immune activation. Nevertheless, folic acid supplementation is able to compensate for the loss of the vitamin due to oxidation. Moreover, oxidative stress may also diminish other antioxidants like vitamins, e.g., α -tocopherol or the aldehyde pyridoxalphosphate and also tetrahydrobiopterin (BH_4), and consequently affect BH_4 -dependent enzymes.

Asp258 an important residue in sepiapterin reductase for substrate binding

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Tetrahydrobiopterin (BH_4) is an essential cofactor for aromatic amino acid hydroxylases in cells. Sepiapterin reductase (SPR) is the important enzyme that catalyses the last step in the biosynthetic pathway of BH_4 , and is important for controlling the level of BH_4 . Significantly, SPR could reduce various carbonyl compounds of non-pteridine derivatives such as phenylpropanedione. Earlier, the crystal structure of mouse SPR in its ternary complex with oxaloacetate and $NADP^+$ was determined. More recently, we determined, by mutational analysis, that the Ser158, Tyr171, and Lys175 (Ser-Tyr-Lys triad) in the rat SPR subunit was the catalytic site (Fujimoto et al., *Biochim. Biophys. Acta*, 1431, 306-314, 1999). As to the pterin binding site, SPR has a pentapeptide sequence (A-X-L-L-S) in its N-terminus, which was found by Citron et al. to be a common amino acid sequence of the putative pterin binding site in all of the known BH_4 -requiring aromatic amino acid hydroxylases. However, we proved that this A-X-L-L-S sequence serves not as a pterin binding site but as a component of the coenzyme binding domain (Fujimoto et al., *Biochim. Biophys. Acta*, 1999). According to the crystal structure of mouse SPR, Asp258 binds to the guanidino moiety of the pteridine substrate. In this study, we employed site-directed mutagenesis to examine the roles of the Ser-Tyr-Lys triad and Asp258 in rat SPR for the native pteridine substrate and carbonyl compounds. Site-directed mutants were constructed for these residues. Ser158 was changed to Asp (S158D); Tyr171, to Val (Y171V); and Lys175, to Ile (K175I). Site-directed mutants of S158D, Y171V, and K175I decreased K_m s for pterin and carbonyl compounds, and showed low activity (k_{cat}/K_m values) of about 25 % of that of the wild-type. The double-point mutant Y171V&S158D was found to be inactive. These results show that Ser158, Tyr171, and Lys175 contributed to the catalytic activity and that especially both Tyr171 and Ser158 are necessary for the catalysis of carbonyl compounds as well as native pteridine substrate. To know the effect of Asp258 on pteridine binding, we changed Asp258 to Ala (D258A) or to Ser (D258S). Site-directed mutants D258A and D258S showed a similar k_{cat} for sepiapterin but their K_m was about two times higher than that of the wild-type enzyme. On the other hand, these mutants showed no change in K_m values for carbonyl compounds. The above results indicate that the Ser158, Tyr171, and Lys175 residues in SPR are the catalytic site for both pterin and carbonyl compounds. Although SPR shows a strict specificity for pteridine substrates, SPR reduces the carbonyl group of non-pteridines. Also, Asp258 is an important residue for

pterin binding, bindings to the guanidino moiety of the pteridine substrate, but not acting as an anchor for non-pteridine substrates.

Urinary neopterin concentrations in children with asthma and infections

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In healthy adults, an inverse relationship was found between serum immunoglobulin E (IgE) and neopterin concentrations. Increased levels of IgE indicate allergic hypersensitivity and activation of Th2-type immune response, whereas increased neopterin concentrations relate to the activation of Th1-type (cellular) immune response and oxidative stress. Urinary neopterin concentrations (related to creatinine content) were measured by HPLC in 45 children with asthma (average age: 59 months), in 29 children with rotavirus infection (24 months), 9 with other virus infections, 5 with bacterial infections and 6 with both types of infections. In addition, neopterin concentrations were measured in 29 healthy controls (average age: 30 months). Average neopterin concentrations in children with asthma did not differ from controls, in contrast, significantly higher neopterin concentrations were observed in children with any type of infection, and there was no difference between different groups of infections, although rotavirus infections seemed to be higher than the other three groups ($p = \text{not significant}$). In children with asthma IgE levels were positively correlated with age ($r_s = 0.540$, $p < 0.001$) and inversely with neopterin levels ($r_s -0.308$, $p = 0.02$), and there was an inverse relationship between neopterin levels and age ($r_s = -0.596$, $p < 0.01$). Neopterin concentrations in children with asthma depended on family history of atopy, family history which was associated with higher levels, but were independent from medication or steroids used. Urinary neopterin concentrations were significantly higher in children with infections compare to children with asthma. The inverse relationship between IgE and neopterin concentrations in children with asthma is in line with the concept of a cross-regulatory interaction between TH-1- and TH2-type immune response.

Nitric oxide synthase is induced in sporulation of *Physarum polycephalum*

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Physarum polycephalum is a plasmodial slime mold. It exists as different forms of live and serves as a model system to study differentiation processes. *Physarum polycephalum* expresses a calcium-independent nitric oxide synthase with biochemical characteristics of mammalian inducible nitric oxide synthase. This first non-animal nitric oxide synthase identified shares less than 39 % amino acid identity with nitric oxide synthases from other species but the binding motifs for all cofactors are conserved and the spacer sequence responsible for calcium dependence is lacking. Here we investigated the role of nitric oxide synthase, GTP-cyclohydrolase I, cGMP and *Lig 1*, a recently discovered early gene which is strongly up-regulated during formation of sporangia, induced 6 to 8 h after light treatment, during the sporulation of the slime mold. Sporulation, a differentiation process, is induced after starvation of macroplasmodia for several days in the dark by a light pulse.

Expression and activity of nitric oxide synthase, as was determined by Northern analysis and by HPLC quantification of nitrite plus nitrate in supernatants from macroplasmodia, were strongly up-regulated during the five day starvation period. In contrast, GTP cyclohydrolase I is down-regulated. GTP cyclohydrolase I in *Physarum* is a key enzyme for two major pteridine biosynthetic pathways, i.e. formation of tetrahydrobiopterin, a NOS cofactor, and of folic acid, which is essential for cell proliferation. While intracellular tetrahydrobiopterin levels, although strongly decreased, are still high enough (~ 500 pmol/mg) at the end of the starvation period, to support NOS activity, this repression of GTP cyclohydrolase I might affect proliferation via decreasing folate availability.

Glucose, a growth signal and known repressor of sporulation, prevented induction of NOS mRNA expression and activity and *Lig 1* expression when maintained at 62 mM, the usual growth medium concentration. Inhibition of NOS by L-N6-(1-iminoethyl)-lysine (NIL) and of NO sensitive guanylate cyclase by 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) suppressed both sporulation and *Lig 1* expression. The effect of NIL could be overcome by 8-bromo-cGMP when added before setting the light pulse (80 % sporulation of NIL/8-bromo-cGMP-treated cultures ver-

sus 0 % of NIL-treated cultures). Our data demonstrate that induction of NOS by glucose limitation is an essential step in gaining sporulation competence of *Physarum* and that NO-mediated cGMP formation is functionally linked to processing the light signal which then triggers phytochrome-induced *Lig 1* expression and sporulation.

Assessment of the immune response in renal transplant patients using neural networks

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Neural network architecture has been successfully applied to various scientific and technological problems. Therefore we have investigated the use of this technique in analysing data obtained by serial measurements of clinical and immunological markers in the postoperative course after kidney transplantation for the differential diagnosis of cytomegalovirus (CMV) infections and acute renal allograft rejections which is crucial in transplant medicine.

In 64 consecutive renal transplant patients the following parameters were measured daily in serum, plasma, and urine during the postoperative stay on ward (p.o.obs. mean = 29.2 days, Σ = 1869 days): Serum and urine neopterin, serum amyloid A (SAA), serum C-reactive protein, and complement C5a in plasma and urine. Tests for herpes viruses (serology, PCR, and early antigen pp65 detection) were performed at least twice weekly. Retrospectively the following clinical situations were defined: Acute rejection episodes (n=26, 104 days), CMV-diseases (n=6, 116 days), and periods of stable graft function (n=26, 104 days). The mean parameter values in these three groups were compared using the Kruskal-Wallis test in order to find out which of the above mentioned markers may be able to distinguish between the different diagnostic categories. The data set of the resulting parameters was used to train a feed-forward artificial neural network using an error back propagation algorithm. In order to judge the potential of neural computing in the chosen setting, the same data were analysed by two additional statistical techniques: The generalised likelihood ratio concept and the classification and regression trees

technique (CART-Analysis). Afterwards the accuracy of the classification reached by the three methods was examined on the basis of defined test-data.

Urine neopterin and SAA were the parameters which differed most significantly between the three groups ($p < 0.001$, Kruskal-Wallis test) and therefore seemed to have the best differential diagnostic ability. Using these two markers the artificial neural network classified 80% of the test-data correctly after the supervised learning process. The binary decision tree obtained by the CART-Analysis led in 90% of the cases to a correct result and the prediction using the generalised likelihood-ratio concept was in 85% successful.

Artificial neural networks appear to be promising in medical classification problems, particularly in situations with complex data structures and multiple diagnostic categories. It should be investigated with more emphasis to become a helpful tool in medical decision making.

7,8-Dihydroneopterin and neopterin interfere with copper-mediated low density lipoprotein oxidation

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Oxidatively modified LDL exerts chemotactic properties on macrophages and facilitates the formation of lipid-loaden foam cells, a hallmark of early stage atherosclerosis. Human macrophages stimulated by interferon- γ generate reactive oxygen species, caeruloplasmin, peroxynitrite as well as neopterin and 7,8-dihydroneopterin. Early studies have provided evidence that pterin derivatives are able to interfere with reactive species. We therefore investigated the modulation of LDL oxidation mediated by peroxynitrite and copper. Recording the formation of conjugated dienes in the course of lipid oxidation, measurement of the relative electrophoretic mobility of oxidised LDL and the estimation of specific epitopes generated on oxidised LDL with the monoclonal antibody OB/04 we found that neopterin increased LDL oxidation rate mediated by peroxynitrite and/or copper, respectively.

7,8-Dihydroneopterin showed an antioxidative effect on the peroxynitrite mediated oxidation of LDL within the first 60 minutes. Later on the antioxidative effect changed to a slight pro-oxidative compared to

peroxynitrite alone. In the case of copper (II) mediated LDL oxidation 7,8-dihydroneopterin exerted an anti-oxidative effect when co-incubated with copper and LDL. A slightly pro-oxidative potential was found after pre-incubation of LDL with 7,8-dihydroneopterin for 20 minutes.

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Influence of copper ions on the stability of 7,8-dihydroneopterin and neopterin during LDL oxidation

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Oxidation of low density lipoproteins (LDL) is considered to be one of the key events in pathogenesis of atherosclerosis. Mainly two processes play essential role in LDL oxidation: (1) lipid peroxidation generating peroxy radicals which interfere with a great number of unsaturated lipids involving molecular oxygen. (2) Concomitant with the lipid peroxidation chemical modification of the apolipoprotein B moiety occurs which has chemotactic properties to monocytes and macrophages. LDL oxidation can be induced by transition metal ions like copper (II). In addition, interferon- γ -activated macrophages are able to generate caeruloplasmin, peroxynitrite and neopterin and 7,8-dihydroneopterin. We investigated the influence of copper (II) on neopterin and 7,8-dihydroneopterin during LDL oxidation.

For the detection of oxidation parameters we recorded the time dependent formation of dienes at A_{234nm} . While neopterin was stable during the incubation with 30 μ M copper (II) without LDL the stability of 7,8-dihydroneopterin decreased dramatically within 30 minutes. 7,8-dihydroneopterin was stabilised by adding 30 μ M EDTA to complex copper (II).

Freshly prepared LDL (0.2mg/mL LDL) of three different donors were used to estimate the formation of dienes during a copper (30 μ M) mediated LDL oxidation in the presence or absence of 100 μ M neopterin and 100 μ M 7,8-dihydroneopterin. Subtracting the blank absorbance of neopterin and 7,8-dihydroneopterin with copper neopterin showed a pro-oxidative effect, while 7,8-dihydroneopterin acted anti-oxida-

tive. Longer storage of these LDL samples at 4°C induced an oxidative modification expressed as the content of generated lipid peroxides (LPO; > 6 nmol LPO/mg LDL). Freshly prepared LDL has nearly 0 nmol LPO/mg LDL. 7,8-dihydroneopterin showed a strong pro-oxidative effect during copper mediated oxidation of moderately oxidised LDL samples (> 6 nmol LPO/mg LDL) compared to freshly prepared LDL.

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Neopterin and cytokine release in ovarian cancer before and after surgery and chemotherapy

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Ovarian cancer is the leading cause of cancer deaths in gynecological malignancies. Due to diffuse symptoms 60-70% of ovarian cancer is detected in advanced stages, III-IV, with a poor prognosis. Tumor markers as diagnostic, prognostic and as monitoring factors are needed. Ovarian cancer is treated by primary cytoreductive surgery and adjuvant chemotherapy. Twenty-four women with ovarian cancer were followed consecutively prospective. Plasma samples were taken preoperatively, 4 weeks after surgery just before the first chemotherapy cure and 20 weeks after surgery just before the sixth cure of chemotherapy. Concentrations of neopterin (nmol/l), interleukin-6 (IL-6) (pg/ml) and terminal complement complex (TCC, SC5b-9) (ng/ml) were determined in plasma. Results are given in mean and range. The follow-up time is 36-48 months.

Women with early stage ovarian cancer (n=9), stage I-II, were compared to women with more advanced ovarian cancer (n=15), stage III-IV. Preoperatively neopterin were higher, 10.9 (3.6 - 17.5) vs 7.8 (6.2 - 11.2) $p < 0.05$, and preoperatively IL-6 were increased, 11.5 (0 - 46.6) vs 6.1 (0 - 36.6) $p < 0.05$, in the more advanced stages. Within the groups IL-6 in stage III-IV, decreased from preoperatively to after the fifth cure of chemotherapy, 11.5 (0 - 46.6) vs 2.9 (0 - 14.1) $p < 0.01$, and in stage I-II a significantly increase in TCC,

from preoperatively as compared to as well 4 weeks postoperatively as after 5 chemotherapy cures, 123 (0 - 255) vs 233 (38 - 518) $p < 0.01$ and vs 210 (125 - 449) $p < 0.05$, respectively. Two deaths in stage I-II and seven deaths in stage III-IV. Women with complete remission after five chemotherapy cures ($n = 18$) were compared to women with residual tumor after 5 chemotherapy cures ($n = 6$). No differences were seen between the groups and not within the group with residual tumor. Within the women with complete remission neopterin decreased from just before chemotherapy as compared to after 5 cures, 10.5 (4.5 - 22.4) vs 8.4 (3.9 - 15.0) $p < 0.01$. IL-6 decreased from preoperatively as compared to after five chemotherapy cures, 9.4 (0 - 46.6) vs 2.0 (0 - 14.1) $p < 0.05$, in women with complete remission. Within the complete remission group TCC increased from preoperatively as compared to 4 weeks postoperatively as well as compared to after 5 chemotherapy cures, 116 (0 - 255) vs 188 (35 - 518) $p < 0.01$ and vs 193 (60 - 444) $p < 0.001$, respectively. Five deaths in complete remission and four deaths in residual tumor group.

Women who survived ($n = 15$) were compared to non-survivors ($n = 9$). Neopterin was increased preoperatively in non-survivors as compared to survivors, 12.4 (6.8 - 17.5) vs 7.7 (3.6 - 11.2) $p < 0.01$. Within the groups neopterin in non-survivors decreased from just before chemotherapy to after 5 chemotherapy cures, 12.6 (6.5 - 22.4) vs 9.4 (4.7 - 16.1) $p < 0.05$, and in non-survivors IL-6 was lower after 5 chemotherapy cures as compared to preoperatively, 3.4 (0 - 14.1) vs 14.4 (1.7 - 46.6) $p < 0.05$. Within the survivors group TCC increased from preoperatively as compared to 4 weeks postoperatively as well as compared to after 5 chemotherapy cures, 108 (0 - 255) vs 181 (38 - 518) $p < 0.01$ and vs 199 (60 - 450) $p < 0.01$, respectively.

In conclusion neopterin seems to be a prognostic factor and could also be a diagnostic marker in ovarian cancer. Neopterin might be used during chemotherapy as a monitoring factor. Interleukin-6 could be a diagnostic factor and a monitoring compound in chemotherapy in ovarian cancer. TCC might be useful as a monitoring factor in chemotherapy treatment of ovarian cancer.

The effect of highly active antiretroviral treatment on serum neopterin concentrations in HIV-infection

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Since 1996 highly active antiretroviral treatment (HAART) has dramatically improved the clinical outcome of AIDS and has lead to immune reconstitution in a large group of HIV infected patients. In the same year the development of commercial quantitative HIV-PCR methods made it possible to determine the viral load. Viral load was measured by Roche HIV amplicor test and serum-neopterin by radioimmunoassay (IMMU test, BRAHMS, Berlin, Germany) in 124 HIV infected patients without ongoing opportunistic infection or antiretroviral treatment. As expected serum-neopterin concentrations were correlated to viral load with the highest level (mean 28.6 nmol/l) in 15 patients with 10^5 - 10^6 HIV-RNA copies/ml, 22.3 nmol/l in 63 patients with 10^4 - 10^5 HIV-RNA copies/ml, 18.9 nmol/l in 38 patients with 10^3 - 10^4 HIV-RNA copies/ml and 9.6 nmol/l in 8 patients with 10^2 - 10^3 HIV-RNA copies/ml. In 17 patients with in mean 40,000 HIV-RNA copies/ml who had a successful effect of HAART on viral load (< 200 HIV RNA copies/ml), serum neopterin decreased from pre-treatment concentrations of in mean 19.6 nmol/l, to 9.8 nmol/l after 3 months HAART, and to 5.0 nmol/l after 12 months of HAART. The decrease was correlated to the decrease in number of HIV-copies/ml plasma but there was a slight delay in decrease of serum-neopterin compared with viral load at the 3 months observation. In 10 patients with virologic failure and 10^3 - 10^5 HIV-RNA copies/ml following HAART, serum neopterin concentrations did not significantly differ from untreated patients in the same viral load interval. This suggests that antiretrovirals do not independently affect the immune stimulation. In HIV-patients without opportunistic infections serum-neopterin might be a cheap alternative to quantitative HIV-PCR methods to follow treatment effects.

Physiological concentrations of L-ascorbic acid lead to a chemical stabilization of tetrahydrobiopterin and an increased nitric oxide synthesis in human umbilical vein endothelial cells

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Human umbilical vein endothelial cells were treated in culture with 1 to 1000 μ M L-ascorbic acid for 24 h. This led to a dose-dependent increase in the formation of tritiated citrulline from tritiated L-arginine, as well as to a dose-dependent parallel increase in intracellular concentration of cGMP. When cells were pretreated with sepiapterin (10 μ M), citrulline formation and cGMP concentrations increased to an extent comparable to L-ascorbic acid. When added to sepiapterin-treated cultures, L-ascorbic acid did not cause an additional effect. To unravel the mechanism of action of L-ascorbic acid on nitric oxide formation, we studied the impact of L-ascorbic acid treatment on tetrahydrobiopterin metabolism in human umbilical vein endothelial cells. L-ascorbic acid led to a dose-dependent, up to three-fold increase in intracellular tetrahydrobiopterin concentrations. GTP cyclohydrolase I activity and expression, as well as 6-pyruvoyl tetrahydropterin synthase activity, however, remained unchanged upon L-ascorbic acid treatment. We then checked biopterin concentrations in cells and in supernatants of cytokine-treated human umbilical vein endothelial cells cultivated for 24 hours with increasing doses of L-ascorbic acid. The sum of all biopterin derivatives in cells and media was not influenced by L-ascorbic acid treatment. While tetrahydrobiopterin inside the cells increased with L-ascorbic acid treatment, the amount of dihydrobiopterin and biopterin released to the culture media decreased to the same extent. These results demonstrate that L-ascorbic acid increases intracellular tetrahydrobiopterin concentrations in human umbilical vein endothelial cells by a chemical stabilisation of tetrahydrobiopterin, thus resulting in increased endothelial cell nitric oxide synthesis.

Monitoring of neopterin in patients undergoing tumor immunotherapy

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Currently, intravesical BCG (*Bacillus Calmette Guérin*) is the treatment of choice for superficial bladder cancer. Metastatic renal cell cancer (RCC) is resistant against chemotherapy. Therefore, dendritic cell-based immunotherapy has become an interesting treatment option. Since these immunotherapies are thought to induce mainly Th1-type immune responses neopterin may be a monitoring parameter. Serum from 28 patients (mean age 57, range 42 - 76; 9 women and 19 men) with metastatic RCC was collected during dendritic cell-based immunotherapy. In addition, morning urine samples were collected daily from three patients. Blood was collected before each vaccination and in the 2 patients with cyclophosphamid pretreatment also before chemotherapy. Furthermore, urine samples of 5 patients (mean age 61, range 54 - 72; 2 women, 3 men) with superficial bladder cancer were examined during treatment with intravesical BCG. Neopterin values (nmol/l) from RCC patients are shown below. Metastatic sites are also indicated.

	Mean	Median		Mean	Median
survival > 6 months	8,9	8,4	Lung	12,9	9,3
survival < 6 months	18	9,4	Liver	12,76	9,5
			Bone	19,5	9,5
No pretreatment	9,9	8,9	Adrenal	22,8	17,3
pretreatment	15,9	11,5	soft tissue	8,9	7,15

A modest increase in neopterin levels could be measured during the vaccination. Urine neopterin did not show a clear correlation with vaccination. Nine of 13 patients who died early presented with increased neopterin levels. In contrast, patients with prolonged survival showed decreasing or at least stable neopterin values.

All patients with bladder cancer showed an increase in urinary neopterin after BCG administration. Neopterin levels were maximal on day 2 to 4 when side effects such as urge and fever occurred. Mean follow up is 1 month.

Consistent with earlier observations, neopterin is useful to monitor the responses of bladder cancer patients to BCG treatment and appears to be a prognostic factor in renal cell carcinoma.

Inhibition of iNOS gene expression in ovarian carcinoma cells by neopterin

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In a number of malignant diseases including ovarian carcinomas, increased serum or urine levels of neopterin serve as a valuable marker for the state of activation of the cellular immune system.

Reported correlations between elevated neopterin concentrations and shorter survival time of the tumor host might be explained by direct biochemical actions exhibited by the pteridines compound. In the present study, we investigated the influence of neopterin on inducible nitric oxide synthase (iNOS) gene expression in two ovarian carcinoma cell lines. Qualitative and quantitative analyses of iNOS as well as measurements of nitric oxide (NO) metabolites in the cell supernatant revealed an inhibitory effect of neopterin (1000 μ M) on NO generation induced by a combination of interferon- γ (100 U/ml), tumor necrosis factor- α (500 U/ml), and interleukin-1 β (10 U/ml). Since NO is most likely involved in apoptotic cell death in ovarian carcinoma cells, it is conceivable that suppression of NO synthesis represents a biochemical mechanism mediated by neopterin that is advantageous to tumor progression and thus harmful to the host.

Monitoring of cytokine activity in cells and cell lines of different species

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The eye as part of the brain is susceptible for neurotropic viruses. Viral infection of retinal pigment epithelial cells (RPE) might play a role in the pathogenesis of autoimmune uveitis. We investigated the following questions: can RPE be infected by various viruses causing common infections in humans and how do RPE cells interact with the immune system? We used the following viruses to infect cultured human RPE: Cytomegalovirus (CMV), Coxsackie virus B3 (CVB), herpes simplex virus type 1 (HSV) and type 6 (HHV-6), Epstein-Barr virus (EBV), adenovirus types 1 and 7, measles virus, influenza A/B virus, Parainfluenza virus, respiratory syncytial virus (RSV), rhino virus and pseudorabies virus. Double staining fluorescence technique was used for visualization of virus infection and different cell surface markers in the same cells and was analyzed by laser microscopy. RPE

cultures could be infected with different neurotropic viruses, respiratory viruses and enteroviruses - whereas no infection was observed with lymphotropic viruses. CMV down-regulated MHC-class I antigens on RPE, whereas CVB and HSV did not alter MHC-class I antigen expression. No induction of class II antigens was observed in RPE infected with CVB, HSV or CMV. Adhesion molecule ICAM-1 (CD54) was slightly increased after virus infection and the other cell surface molecules did not alter. Although we were not able to detect macrophage marker CD68 on our RPE cultures, RPE and macrophages showed several similarities. Using fluorescent labeled synthetic microparticles, FITC-labeled staphylococcus aureus or transgenic green fluorescent protein (GFP)-expressing E.coli, a clear size-dependent uptake of particles was seen in RPE as well as in a RPE-cell line (CRL) in comparison to macrophages and macrophage cell line (Mono Mac6). Opsonisation with serum or purified complement factors did not lead to increased phagocytosis and also the classical complement-receptors (CR1, CR3) were not found on RPE.

IFN- γ has been shown to induce neopterin-production by monocytes, we therefore investigated its effects on RPE. In contrast to macrophages and Mono Mac6 no induction of neopterin was observed on RPE or CRL by IFN- γ (100 - 10.000 U) while tryptophan degradation was always observed in CRL - thus indicating major differences of RPE and macrophages. Although there is no neopterin-release in RPE, de novo expression of MHC class-II and up-regulation of class-I has been described on RPE cells upon stimulation by IFN- γ . This indicates that various cytokines and infection mediators might play a role in "autoimmune" retinal disease.

Differentiation of cytomegalovirus syndromes and diseases using neopterin measurements and epitope-specific IgM Western blotting

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Human cytomegalovirus (HCMV) infections remain to be a major cause of morbidity and mortality in patients with a compromised immune system despite remarkable advances in diagnostic tests and

antiviral therapies. The purpose of the underlying study was to investigate the humoral and cellular immune response against HCMV in renal transplant patients by measuring the macrophage product neopterin and IgM antibodies against HCMV specific epitopes using a recently developed western blot.

19 renal transplant recipients who experienced either an HCMV syndrome or an HCMV disease during their post-transplant course were included in this long-term study covering in the mean 942 days of observation. An HCMV syndrome ($n = 6$) was defined as laboratory evidence and clinical signs/symptoms of HCMV infection, an HCMV disease ($n = 13$) was defined as additional organ manifestations of viral infection and the administration of ganciclovir therapy. The Western blot (WB) was developed in our laboratory using recombinant antigens of immunodominant epitopes of the viral tegument proteins pp65 (gpU83) and pp150 (gpUL32) and the glycoproteins gB (gpUL55) and gH (gpUL75) for the detection of HCMV specific IgM and IgG antibodies. Neopterin was determined by an ELISA method (BRAHMS, Berlin, FRG). Neopterin was measured daily in serum and urine during the patient's stay on ward and on each single visit to the outpatient clinics. The WB was measured retrospectively in a total of 305 stored plasma samples.

All HCMV episodes occurring in the 19 renal transplant patients were associated with a detectable change in the WB assay, either by the first appearance of IgM antibodies or an increase in the number of recognized viral epitopes. The humoral immune response occurred in the mean 30 days after the first clinical signs or symptoms were seen and an increase in neopterin appeared. Mean and median serum and urine neopterin values differed significantly between HCMV syndromes versus diseases ($p < 0.01$). In patients with HCMV syndromes glycoprotein-specific IgM was not detectable. In 17 of the 19 patients the IgM response against HCMV persisted over the whole period of observation, i.e. longer than 3 years.

The combined use of neopterin and a specific HCMV test like the WB permits not only a reliable detection of HCMV infections but also an assessment of their severity. This approach facilitates decision making in regard to initiation and duration of anti-viral therapies.

Serum neopterin and homocysteine in patients with dementia

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In earlier studies we have shown increased homocysteine concentrations in the plasma of patients with Alzheimer's disease and other forms of dementia. Homocysteine is considered as a novel risk factor for vascular events and hyperhomocysteinemia was found to affect cognitive function in patients. The conversion of homocysteine to methionine depends on the presence of methyl-tetrahydrofolate, and thus hyperhomocysteinemia is commonly believed to result from insufficient dietary intake of folic acid. In 74 patients with Alzheimer's disease (NINCDS - ADRDA criteria for Alzheimer's disease, 55 females, 19 males, aged 77.4 ± 9.5 years) and 24 controls (10 females, 9 males, aged 74.9 ± 8.2 years) we compared serum homocysteine concentrations (measured by FPIA, Abbott) with folic acid and vitamin B12 concentrations (RIA, Chiron) and with neopterin levels (ELISA, BRAHMS). Homocysteine ($p = 0.03$) and neopterin concentrations ($p < 0.01$) were higher in patients than in controls. Significant correlations existed between homocysteine and folic acid concentrations ($r = -0.3$, $p < 0.05$) and between neopterin and homocysteine ($r = 0.3$, $p < 0.01$). The findings suggest that peripheral immune activation and oxidative stress, as indicated by elevated neopterin concentrations, and biomarker of vascular risk homocysteine may contribute to the pathogenesis of Alzheimer's disease. Moreover, the data imply that oxidative stress could be involved in the development of hyperhomocysteinemia via an increased oxidative degradation of the reductant tetrahydrofolate. In this way folate deficiency may develop despite normal dietary intake of the vitamin.

Neopterin concentrations in colon dialysate

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Elevated concentrations of neopterin indicate activated cellular immunity. The activity of the cellular immune system in the intestinal tract is at present practically not investigated. Thus far, neopterin concentra-

tions have only been measured in stool of 3 cancer patients and of 2 patients with duodenal ulcer. However, the immune system requires an enormous effort to protect the mucosal area in the human intestinal tract which extends to 300 m² (for comparison, the skin as the external surface of the human body covers only 2m²). There are 1011/ml bacteria in the colon and there exist 400-500 potentially harmful species of bacteria. Therefore, it is not surprising that 75% of the cells of the human immune system exist within the intestinal tract and of these 70% within the human colon.

In 27 healthy subjects, in 41 patients with chronic and in 5 patients with very mild acute illness and in 2 patients with acute gastritis we measured urinary neopterin by HPLC, and in the same subjects neopterin concentrations were determined in salivary fluid and in fecal water by RIA. Neopterin concentrations in urine and saliva were found higher in all patients compared to healthy subjects. However, neopterin concentrations in fecal water were found significantly lower (mean: 28.33 mmol/l) than in healthy subjects (71.34 mmol/l), by contrast they were high in faecal water of patients with acute illness and extremely high in that of the 2 patients with nervous gastritis. From the lower neopterin concentrations observed in patients compared to controls, the question arises, whether they are due to a lower neopterin production or by a lower thickening of stool which is in general by a factor of 100. In the same samples, we have also measured 6 short-chain fatty acids. Five of these did not differ significantly concerning the mean percentages whereas the absolute values were different compared with those of controls. In the alkaline pH of the stool, these fatty acids are present as anions and together with the corresponding cation, they represent the majority of the soluble components in fecal water. If the neopterin concentrations of the chronically ill and of the healthy subjects were related to the relatively stable concentration of the short-chain fatty acids, the concentration of neopterin in healthy subjects still remained significantly higher than in the chronically ill patients ($p < 0.01$, ANOVA). Further studies are required to clarify whether neopterin in faecal water results from neopterin transfer from the liver or rather it is produced from immune cells present in ileum and colon.

Serum neopterin concentrations in individuals with and without *Helicobacter pylori* infection

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In order to find out whether chronic immune stimulation is associated with *Helicobacter pylori* infection we studied 425 healthy individuals (aged 57.6 ± 9.22 ; mean $\pm$ S.D.) who were performing a health check up without having any specific health problems. Blood was drawn for *H. pylori* antibody testing, determination of routine laboratory parameters, differential blood count and measuring neopterin concentrations. In addition, in individuals seropositive for *H. pylori*, measurement of ¹³CO₂ exhalation after administration of 75 mg ¹³C-urea was performed to test for the presence of these microorganisms in the gut. One hundred and ninety subjects were *H. pylori* seropositive (antibody titer > 4) and they had higher serum neopterin concentrations (serum neopterin: $6.38 \text{ nM} \pm 3.3 \text{ S.D.}$) compared to seronegative subjects (serum neopterin: $5.74 \pm 2.7 \text{ nM}$, $p = 0.027$, Student's t-test). Among the *H. pylori* seropositive individuals no such difference existed between breath test positive (serum neopterin: $6.1 \pm 3.1 \text{ nM}$) and negative ones (serum neopterin: $6.1 \pm 2.0 \text{ nM}$; $p = \text{n. s.}$).

Increased production of neopterin is indicative for an activated cellular immune response. Antibody seropositive patients present with higher neopterin levels than seronegatives even when *H. pylori* seemed to be no longer present in the gut. Surprisingly neopterin concentrations were not related to the presence/absence of bacteria in the gut. Higher neopterin concentrations in *H. pylori* seropositive individuals would be in good agreement with the view of *H. pylori* infection as a risk factor for developing atherosclerosis, since in earlier studies carotid stenosis was found to be associated with higher neopterin concentrations. In that study as in ours, the great majority of neopterin concentrations found were well within the normal range of healthy controls. Our data point to a role of *H. pylori* to induce a long-lasting albeit moderate deterioration of the cellular immune system, which may contribute to increase the risk of atherosclerosis.

Do *Physarum* mutants with altered sporulation behavior show altered tetrahydrobiopterin-nitric oxide-cGMP metabolism?

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The acellular slime mold *Physarum polycephalum* grows as macroplasmidia on solid surfaces, or as microplasmidia in liquid culture. Macroplasmidia are mobile single giant cells with multiple nuclei, which show the phenomenon of synchronized nuclear division. These macroplasmidia can form sporangia and release spores. Sporulation requires a 5-day starvation period, followed by a 30 min light pulse as a trigger. In previous work, we have shown that a calcium-independent nitric oxide synthase is induced in the starvation period required to achieve sporulation competence. Experiments using drugs to influence nitric oxide/cGMP metabolism suggested a functional role of nitric oxide and cGMP in sporulation of *Physarum polycephalum*. Mutants of *Physarum* with altered sporulation behavior were kindly provided to us by Wolfgang Marwan, University of Freiburg, Germany. These mutants had been characterized for functional differences in sporulation behavior, but the biochemical basis of their altered behavior remained unknown.

In the present work, we investigated these mutants for alterations in tetrahydrobiopterin, nitric oxide and cGMP metabolism during sporulation. Five different mutants, i.e. CS114, which does not sporulate, CS104, which does not react to blue light, CS245, CS602 and CS902, which sporulate without a light pulse, were compared to the parent wild type strain CS310. All strains showed comparable intracellular concentrations of tetrahydrobiopterin, dihydrobiopterin, biopterin and 6-hydroxymethylpterin. Nitric oxide synthase activity was demonstrated in all strains using the citrulline assay, and was found significantly lower in the non-sporulating strain CS114. Upon starvation, all strains showed induction of nitric oxide synthase as demonstrated by Northern blot analysis and accumulation of nitrite and nitrate in the culture media, which was measured by HPLC. Both, induction of nitric oxide synthase and nitrite and nitrate accumulation were significantly higher in the non-sporulating strain CS114 as compared to the other strains. cGMP was measured by radioimmunoassay during the starvation period as well as 6 hours after the light pulse trigger. cGMP levels were constant during the starvation period, and showed a definite increase following the light pulse with the exception of the non sporulating strain CS114, where intracellular cGMP concentrations remained low even after the light pulse. These results show that the mutants investigated have no defects in their capacity to synthesize tetrahydrobiopterin or nitric oxide, that might explain their altered sporulation behavior. The non-sporulating strain CS114, however, shows a significantly altered pattern of nitric oxide synthase induction, and is unique in that intracellular cGMP concentrations do not rise in response to the light pulse

triggering sporulation.

Arterial hypertension and immune activation in chronic mesangioproliferative glomerulonephritis

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Kynurenine and neopterin are markers of immune stimulation in humans. To find a possible relationship between markers of immune activation and arterial hypertension we examined 70 patients with chronic mesangioproliferative glomerulonephritis and 10 healthy individuals. In all of our patients and healthy individuals we examined the kidney functions by serum concentrations of creatinine and glomerular filtration rates. We performed the L-tryptophan peroral loading test with L-tryptophan 30 mg/kg body weight. We have determined serum tryptophan, serum kynurenine and serum neopterin 3 hours after L-tryptophan load and also in fasting state. In all patients we twice examined urine and measured the blood pressure before and 3 hours after L-tryptophan load. The mean concentration of kynurenine in patients with MesPGN with proteinuria > 2 g/24 h did not differ from that of healthy individuals. Increased serum concentration of kynurenine at the 3rd hour after L-tryptophan load have been found in 23 patients (38,3%) of MesPGN with proteinuria < 2 g/24 h or in 32.9% of all chronic mesangioproliferative glomerulonephritis patients. In 12 patients (20%) with MesPGN with proteinuria < 2 g/24 h the increased serum concentration of kynurenine has been determined only at the fasting state. The concentration of serum neopterin in patients with MesPGN with proteinuria > 2 g/24 h before and after L-tryptophan load did not differ from that in the control group. In patients with MesPGN with proteinuria < 2 g/24 h we have detected a positive correlation between serum concentrations of kynurenine and neopterin both, at fasting state and 3 hours after L-tryptophan load. We detected arterial hypertension in 48.6% of patients with hyperkynureninemia and only in 17.1% of patients without hyperkynureninemia. We have found positive correlations between mean arterial pressure and serum concentrations of kynurenine and neopterin. The data suggest that the immune activation in mesangioproliferative glomerulonephritis accompanied by elevated serum concentration of kynurenine and neopterin can contribute to the development of arterial

hypertension.

Impact of folate substitution on homocysteine and neopterin levels in hemodialysis patients

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Homocysteine is associated with atherosclerosis and enhanced cardiovascular risk. A majority of patients with end-stage renal disease have elevated homocysteine levels. The hyperhomocysteinemia characteristic of dialysis patients is largely refractory to supplementation with B-vitamins and/or supraphysiologic doses of folic acid. The underlying study investigates whether high doses of reduced folates provide a more effective lowering of homocysteine levels in patients on chronic hemodialysis.

Patients and methods: In a double-blind, placebo-controlled, multi-center study 141 hemodialysis patients were randomized into 4 groups according to gender and 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism. For a period of 6 weeks the patients received a daily oral dose of either 7.5 mg of folic acid (PGA, group 1), or 15 mg of D,L-formyltetrahydrofolate (FTHF, group 2), or 15 mg of D,L-methyltetrahydrofolate (MTHF, group 3) or 7.5 mg of a placebo (PLAC, group 4). All patients continued their pre-existing baseline supplementation regimen of 1 mg folic acid, 10 mg vitamin B6 and 6 µg B12 throughout the study. Before, during and after completion of the study (ie. at 0, 3 and 6 weeks) total homocysteine, total folate, vitamin B6, vitamin B12, neopterin, cholesterol, HDL-cholesterol, and triglycerides were measured in plasma immediately before start of hemodialysis treatment. Altogether 120 patients completed the study. Before treatment a majority of hemodialysis patients showed mild hyperhomocysteinemia despite increased plasma folate levels (mean level of homocysteine = 33 µmol/L, mean level of folate = 75 nmol/L). The vitamin B6 levels were also slightly elevated (mean level of 23 µg/L). The mean levels of cholesterol, HDL-cholesterol and triglyc-

erides were 178, 36, and 216 mg/dL, respectively. Neopterin was markedly elevated with an average value of 153 nmol/L. 11 per cent of the patients were homozygous for the MTHFR gene polymorphism. The mean percentage reductions of the homocysteine levels were not significantly different between the treatment groups despite enormous increases of the circulating plasma folate levels. The homocysteine-lowering effect was stronger the higher the homocysteine levels were before starting the treatment with supraphysiologic folate doses, however this trend was not statistically significant and did not result in normalization of the homocysteine levels in the patients. The neopterin values did not show a characteristic pattern during the treatment period.

High-dose oral folate supplementation does not normalize homocysteine levels in hemodialysis patients. Reduced folates seem to have a comparable homocysteine-lowering efficacy than folic acid. .

On the possible relationship between immune activation and dehydroepiandrosterone production

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Dehydroepiandrosterone (DHEA) and its sulfated metabolite DHEA sulfate (DHEAs) are hormones secreted by the adrenal cortex in response to adrenocorticotropin. DHEA is the most abundant adrenal steroid and has only mild androgenic properties. DHEAs is inactive and converted peripherally to DHEA, which exerts biological activity. Recently, it has been clarified that humans largely depend on peripheral tissues for androgen and estrogen formation from the adrenal precursor DHEA and DHEAs.

The age-related decline in dehydroepiandrosterone production is currently attracting attention because of its possible relevance to the etiology and management of a number of age-related clinical disorders. Various abnormalities of immune system function have been described in the elderly. Among them, increased concentrations of neopterin have been reported, which is produced by human monocytes/macrophages upon stimulation by the Th1-cell cytokine interferon-γ.

In order to examine the relation of serum DHEA to serum neopterin, we studied 281 otherwise healthy outpatients, who visited the physician's office for a

medical health check-up. 10 % presented with increased neopterin concentrations, 0.4 % had increased DHEAs concentrations. DHEAs concentrations were significantly higher in patients with lower neopterin concentrations (Mann-Whitney test: $U = 4793$, $p < 0.0001$). There existed a rather strong inverse correlation between DHEAs concentrations and serum neopterin concentrations (Spearman's rank correlation: $r_s = -0.221$, $p < 0.0001$). In agreement with earlier studies males showed higher DHEAs concentrations (median: $4.02 \mu\text{mol/l}$) than females (median: $2.75 \mu\text{mol/l}$; Mann-Whitney test, $U = 4829$, $p < 0.0001$) and an inverse correlation (Spearman's rank correlation: $r_s = -0.541$, $p < 0.0001$) of age versus DHEAs concentrations was found.

The data may support the concept that the decrease of DHEA with increasing age is related to immune system activation. Oxidative stress which accompanies immune response may diminish DHEA synthesis. On the other hand, DHEA could also be able to influence immune system activation. A relative deficiency of DHEA might even contribute for Th1-cell activation and enhanced neopterin production.

About the influence of various emotional states on the dynamic course of urine neopterin in a healthy proband

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Using an "integrative" single-case approach we have been able to demonstrate in recent studies on three patients with systemic lupus erythematosus (SLE) that negative stressful incidents were causally related with an increase in urine neopterin 36 hours (Case 1) to 60 hours (Cases 2 and 3) later. Furthermore, in Cases 2 and 3 it turned out that stressful incidents led to a decrease in urine cortisol 36 hours later. The current study was conducted to attain first evidence about dynamic interdependencies between psychological and biological variables in a healthy person.

A 26 year-old healthy woman collected all of her urine for a period of 63 days on a 12 hour basis for the determination of various biochemical parameters including neopterin. Additionally, the proband

answered questions about her emotional state and life style every 12 hours. Every week she was interviewed using the Incidents and Hassles Inventory (IHI, Brown and Harris). Statistical analysis of the 126 serial values was performed using time-series analysis according to Box and Jenkins.

Preliminary cross-correlation analysis revealed that in our healthy proband the course of neopterin was significantly influenced by various emotional states. For example, whenever the proband felt anxious and depressed urine neopterin increased 12 hours later (lag 1: $+ 0.384$; $p < 0.05$) and then decreased after a total of 24 hours (lag 2: $- 0.395$; $p < 0.05$). Moreover, whenever the proband was mentally active, eager and energetic urine neopterin decreased 24 hours later (lag 2: $- 0.178$; n. s.) and then increased after a total of 48 hours (lag 4: $+ 0.336$; $p < 0.05$).

In conclusion, our results on a healthy woman validate the idea of "integrative single-case studies" as a new approach in psychosomatic research.

Interactions of 7,8-dihydroneopterin with copper: stability and radical formation

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Besides being an antioxidant, 7,8-dihydroneopterin was shown to promote the formation of hydroxyl radicals in the absence of other radical sources. This radical formation is dependent on the presence of transition metal ions and has been investigated in detail for iron ions. As copper ions are important for the oxidative modification of low density lipoprotein and copper could be available physiologically from caeruloplasmin in the vicinity of macrophages, we investigated the influence of copper ions on the stability of dihydroneopterin and the formation of hydroxyl radicals.

For the detection of hydroxyl radicals aromatic hydroxylation of salicylic acid was used. Detection and quantification of dihydroneopterin and hydroxylation products was achieved by HPLC with electrochemical detection.

Copper ions dramatically decrease the stability of dihydroneopterin. The main oxidation product is dihydroxanthopterin, just as is the case with iron ions. The consumption of dihydroneopterin is paralleled by the formation of hydroxyl radicals. The breakdown of dihydroneopterin is caused by the copper ions and not

by the radicals formed. Contrary to iron ions, chelating the copper ions completely inhibits their effect on both, dihydroneopterin consumption and radical formation. Furthermore, in contrast to iron ions already minute amounts of copper ions like those found as impurities in buffer solutions are sufficient to decrease the stability of dihydroneopterin.

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Are elevated serum procalcitonin, C-reactive protein, and neopterin levels predictive markers in the diagnosis of infection in neonates?

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Infection in early life is a major cause of mortality and morbidity. The symptoms and signs of sepsis in premature newborns are often non-specific, making diagnosis difficult and the optimal strategy for managing these infants unclear. Hematologic parameters and other laboratory tests (e.g. acute-phase proteins) sometimes do not permit a reliable diagnosis of sepsis. It can take days before microbiological results are available.

During the period from September 1999 to January 2001 charts of 49 newborns with perinatal infection (700-3800 g, gestational age 25 - 42 weeks) were analyzed according to the dynamics of C-reactive protein (CRP), procalcitonin (PCT), and neopterin in the diagnosis of infection. In parallel, CRP, PCT, and neopterin were determined also in the umbilical cord blood of 104 healthy term neonates. CRP was measured ultrasensitively by nephelometry (normal concentration < 1,7 mg/l), PCT by quantitative immunochemiluminescence determination (normal range of PCT in neonates < 0.5 ng/ml, severe bacterial infection > 2ng/ml), neopterin by ELitest (normal range of neopterin in neonates < 65 nmol/l). PCT and neopterin tests were from BRAHMS-Diagnostica (Henningsdorf/Berlin, Germany). Concentration ranges in the umbilical cord blood of healthy term neonates were for PCT: 0.001 -

3,813 ng/ml (mean $\pm$ S.D.: 0.24 ng/ml $\pm$ 0.495), neopterin: 8.61 - 28.4 nmol/l (16.2 nmol/l $\pm$ 4.72), CRP: 0.16 - 1.34mg/l (0.16mg/l $\pm$ 0.16). The levels of PCT, neopterin and CRP in the group of neonates (n=49) with infection were: PCT 0.001 - 132 ng/ml (6.21ng/ml $\pm$ 18.76), neopterin: 3.42 - 240 nmol/l (45.3 nmol/l $\pm$ 28.60), CRP: 0.16-228.0mg/l (22.53mg/l $\pm$ 46.21).

The dynamics of PCT, neopterin and CRP are illustrated by some case reports: Newborn #1: from high risk pregnancy, premature rupture of membranes (48 h) mother CRP positive (80mg/l), treated for candidosis, (vaginosis), spontaneous delivery in 32 gestational week, birth weight 1500, Apgar score: 8/7/9. Respiratory support (nasal CPAP) for 3 days laboratory parameters leukocytes 35,000 /mm³, initial treatment of antibiotics changed to antimycotic therapy (after 5 days according to the results from microbiology: -Candida albicans), day 15 worsening of the clinical state - respiratory problems, low saturations due to infection CRP 23mg/l, PCT 24ng/ml, neopterin 52nmol/l. The high levels of neopterin the first 12 days might be caused by mycotic infection in the baby. Newborn #2: from high risk pregnancy, positive urine culture in the mother, treated with antibiotics, delivery in 36th gestation week - per cesarean section because of hypertension and disturbances in fetoplacental circulation, birth weight 2800g, Apgar score: 7/9/10, immediately after birth respiratory problems (nasal CPAP-3 days), on X-ray pneumonia. Laboratory parameters did not significantly support the diagnosis of infection in this newborn. Newborn #3: from high risk pregnancy, drug abuse 4 years heroin, 2.5 years in Methadon program, delivery at home, brought to hospital by ambulance, birth weight 1670 g, hypothermia, gestational age 32 weeks, respiratory problems, resuscitation, in spite of GBS infection, all parameters CRP, PCT without elevation, only slight depression of leukocytes on the second day of life. In the 5th week of life respiratory problems due to viral infection. During this period PCT and CRP were low and only neopterin remained elevated. Newborn #4: Positive urine culture in mother, treated with antibiotics, positive GBS culture, delivered spontaneously in the 40th gestational week, birth weight 2800g, Apgar score: 10/10/10, on the second day of life clinical signs of severe infection with elevated CRP, leukocytes and positive lumbar puncture in spite of negative blood culture. This represents a relatively paradox situation when a newborn with severe infection had very high level of CRP without elevation of PCT, but also high neopterin level. None of the parameters (CRP, PCT, neopterin,) alone is a reliable marker of infection in neonates, only combination of them and their dynamics together with the

clinical picture can help in the diagnosis of infection in newborns; routine daily examination of CRP, PCT, and neopterin is recommended.

Neopterin expression in trauma patients

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The physiologic acute response to a traumatic injury represents a balanced, finely tuned interreaction among the nervous, endocrine and immune systems. This early, controlled inflammation is supposed to restore homeostasis and induce adequate healing processes in the injured organism. An exuberant inflammatory response may lead to a counterregulatory immunosuppression that results in bacteremic sepsis (BS), adult respiratory distress syndrome (ARDS), multiple organ failure (MOF) and death. BS and MOF are a significant cause of death in trauma patients after successful resuscitation. Reliable, early sensitive indicators of inflammation and oxidative stress may help identify patients at risk for BS, ARDS, MOF and death. When recognizing foreign antigens, T-lymphocytes release interferon- γ , which stimulates the production and release of neopterin by human macrophages. Interferon- γ induced expression of neopterin by macrophages correlates with the extent of immunologically induced oxidative stress. Tumor necrosis factor- α (TNF- α) enhances interferon-induced neopterin production. Elevations of TNF- α have been reported in patients with fatal BS. Neopterin expression can thus be viewed as the final common pathway of all pro- and counterinflammatory reactions of the monocyte/macrophage system in response to interferon- γ stimulation after trauma. We hypothesized that elevated neopterin levels indicate a hyperimmune response that culminates in BS, ARDS and MOF. We further hypothesized that protracted elevations of neopterin indicate continued challenge of the immunologic system in trauma patients. Neopterin levels were assayed by ELISA (lower limit of sensitivity 2 nmol/l in serial plasma specimens from 22 adult trauma patients. Neopterin levels were elevated in all patients

from the time of admission and tended to normalize by the end of the first week after injury. Septic patients had significantly higher neopterin levels on day 1 (19.6 ± 3.8 nmol/l vs. 8.4 ± 0.9 nmol/l; $p = 0.04$) and day 2 (21.3 ± 4.6 nmol/l vs. 8.5 ± 1.3 nmol/l; $p = 0.05$). Patients with ARDS also had significantly higher levels on day 1 (23.8 ± 2.9 nmol/l vs. 9.0 ± 1.1 nmol/l; $p = 0.02$) and day 2 (27.7 ± 2.5 nmol/l vs. 8.9 ± 1.3 nmol/l; $p = 0.007$).

As expected, neopterin levels were elevated above normal in all trauma patients on admission. Neopterin levels remained at high normal to slightly elevated levels in trauma patients who had an uneventful hospitalization. However, neopterin levels were increased to greater than twice normal in patients who developed BS, ARDS and MOF. This elevation correlated well with an increase in TNF- α levels, confirming the immunological challenge. The highest TNF- α and neopterin levels were obtained from a patient with a missed colon injury who expired on postinjury day 10. Neopterin levels remained highly elevated in patients with BS and ARDS as an expression of the ongoing stimulation of the immune system (either from an exuberant reaction to the trauma per se or from ongoing stimulation from a missed injury) that may ultimately lead to MOF.

Early neopterin levels may be useful to stratify patients into high/low risk categories for BS, ARDS and MOF. Serial neopterin levels in trauma patients may help to determine the need for further investigations or the need for exploration of a patient with a question of missed intra-abdominal injury.

In vitro pyrogen testing - a comparison of possible test systems

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Pyrogen testing is currently performed in rabbits by measuring the possible rise of body temperature after the application of the test drug. As the animals are stressed by the procedure, an impact on the fever reaction can not be excluded. For this reason as well as for economical and ethical concerns several in vitro methods are now evaluated for their potential to identify pyrogenic contamination. On the one hand, assays based on cultivated permanent monocytic cell lines (THP-1 bulk line and clones and MM6) have been developed, on the other hand pyrogen tests using

human whole blood or peripheral blood mononuclear cells (PBMC) have been established. In all these test systems cytokines such as TNF- α , IL-1 β and IL-6 or the pteridine neopterin serve as read-outs which can be detected by ELISA. Currently, the sensitivity and robustness as well as the intra- and interlaboratory variability of the test systems are investigated by applying known amounts of endotoxin (the most common pyrogen). The data presented here are mainly obtained from the cell-based pyrogen test using the human monocytoid cell line THP-1. The cells release neopterin to the culture medium as a response to pyrogenic contamination. This effect is strongly increased by costimulation with IFN- γ . In contrast to earlier experiments where a highly significant correlation between formation of neopterin and TNF- α could be shown, the increase of cytokine rates of IL-1 β , IL-6 and TNF- α in the supernatant could be detected by the ELISA systems used for these experiments only when the cells were stimulated with high endotoxin doses. Similar results were obtained when the amount of cytokine (IL-1 β and IL-6) as well as chemokine (MIG, IP-10, CXCL11) and GTP-cyclohydrolase I mRNA was determined by quantitative PCR. Preliminary experiments showed induction of IL-1 β , IL-6 as well as IP-10 mRNA with increasing doses of endotoxin in presence of human IFN- γ . On the other hand, in the human whole blood systems no increase of neopterin could be found which may be due to the small number of neopterin-forming cells in this assay system. Comparison of the whole blood assay and the cell culture assay showed, that the two tests yield comparable results at higher endotoxin concentrations. However, at doses between 7 and 60 pg/ml sample concentration, the cell test requires further optimisation. Future investigations will show whether one of the models is superior to the others or which combination is the most promising to reduce or replace the rabbit pyrogen test and to guarantee the safety of drugs released for parenteral application.

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7,8-Dihydroneopterin and free radicals - a short overview of a fascinating story

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Since more than one decade, numerous experiments have been published proving that pteridine derivatives can exert quite diverse effects upon reactions involving free radicals. While aromatic pterins were found to have pro-oxidative, antioxidative or no effects depending on the system studied, reduced pterins were generally found to rapidly (i.e., within minutes) suppress free radical-mediated reactions in systems involving free radical-generators such as, e.g., hydrogen peroxide, chloramines or even carbon-centered free radicals. However, some authors noted also indirect evidences for pro-oxidative actions of high concentrations of reduced pterins like 7,8-dihydroneopterin. These apparent contradictory findings could be resolved by the demonstration that 7,8-dihydroneopterin (as a typical example) was able to lead to a slow (i.e., lasting for several hours) production of superoxide radical anions and other species like hydroxyl radicals in air-saturated solutions. Detailed experiments showed that the reduced pterin in these reactions functions as ultimate electron source needed to reduce dioxygen, and that transition metal ions such as iron ions are able to greatly influence the kinetics of these reactions via well-known Fenton-type reactions. In fact, ferrous iron reduces the dioxygen molecule to superoxide radical anion, and the resulting ferric iron is reduced to ferrous state by the reduced pterin, the amount of which therefore controls the total amount of free radical yield.

Bearing in mind that activated human monocytes/macrophages produce 7,8-dihydroneopterin and neopterin in an approximate ratio of 2:1, it seems well conceivable that these cells strongly enhance their aggressive potential via the radical-generating capability of 7,8-dihydroneopterin in the presence of dioxygen. Moreover, aromatic neopterin has been shown to act pro-oxidatively in several experiments, and thus may augment the radical-formation by angry macrophages. Importantly, macrophages lack the enzyme myeloperoxidase which enables formation of hypochlorite by granulocytes, and human macrophages also do not produce sufficient tetrahydrobiopterin and, hence, nitric oxide, as macrophages of other mammals generally do. Thus, the described effects of neopterin and 7,8-dihydroneopterin could well provide a reasonable explanation for the hitherto poorly understood production of these compounds by activated human monocytes/macrophages.

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Homocysteine, pyridoxal-5-phosphate deficiency and neopterin in patients with coronary heart disease

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Pyridoxal-5-phosphate (P-5-P) deficiency, increased homocysteine and neopterin concentration in blood serum or plasma are factors acknowledged to participate in the development and progression of coronary heart disease (CHD). To examine the frequency of these biochemical changes in 61 patients with CHD verified by coronary angiography was the aim of this study. L-tryptophan load test followed by kynurenine determination in blood serum has been used for verification of P-5-P deficiency. Homocysteine concentration in blood serum has been identified by the method of Abbot IMx, while for serum neopterin determination the method of radioimmunoassay has been used. We have found P-5-P deficiency in 78 % of observed patients with CHD, while elevated homocysteine concentration was identified in 32.8% and increased neopterin concentration was observed in 20% of cases. Although all enzyme systems which convert homocysteine to cysteine are P-5-P dependent our results show that in CHD patients the frequency of P-5-P deficiency is higher than that of elevated homocysteine concentration in serum. However in 20% of CHD patients we have observed both increased neopterin concentration and P-5-P deficiency there are no data in literature about the relationship between these indexes.

Changes in blood cell counts in patients treated with antiretroviral therapy.

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A decrease in blood cell counts (leukocytes, granulocytes, thrombocytes) is frequently seen in HIV

infected individuals. Thrombocytopenia is well known as a clinical manifestation of mild symptomatic HIV infection (stage B of HIV infection). Highly active antiretroviral treatment (HAART) is associated with a decrease in immune activation, (including decreased levels of neopterin), an increase in CD4⁺ T cell counts and a decrease in HIV-1 RNA. In this study we addressed the question: is there a significant change in blood cell counts (leukocytes, granulocytes, thrombocytes) during antiretroviral therapy? If so, is the decrease of immune activation and HIV-1 RNA and the increase in CD4⁺ T cell counts in antiretroviral treated patients associated with the changes in blood cell counts? To answer this question, we evaluated treatment naive patients during the first six months of antiretroviral therapy for the change in levels of immune activation markers (neopterin, sTNFR II), CD4⁺ T cells, HIV-1 RNA and blood cell counts (leukocytes, granulocytes, lymphocytes, thrombocytes). Patients treated with growth factors (G-CSF) or thrombocyte transfusions were excluded from the analysis.

55 individuals were included in the study, 16 women and 39 men, mean age was 38.4 ± 9.6 years, 31 had AIDS. During the first 6 months of HAART the CD4⁺ T lymphocytes increased from median 81 cells/ μ l to 220 cells/ μ l, median HIV-1 RNA levels decreased from 5.4 log₁₀ copies/ml to 2.22 log₁₀ copies/ml and median serum neopterin levels decreased from 25 nmol/l to 8 nmol/l (for all, $p < 0.001$). Leukocyte counts increased from baseline counts of $4.2 \cdot 10^3/\mu$ l to $6.1 \cdot 10^3/\mu$ l, lymphocyte counts from $1.0 \cdot 10^3/\mu$ l to $1.5 \cdot 10^3/\mu$ l, thrombocyte counts from $129 \cdot 10^3/\mu$ l to $225 \cdot 10^3/\mu$ l (for all $p < 0.001$) and granulocyte counts from $2.3 \cdot 10^3/\mu$ l to $3.0 \cdot 10^3/\mu$ l ($p = 0.002$). Thrombocyte counts at baseline and the change of thrombocyte counts during therapy correlated only with HIV-1 RNA at baseline ($r = -0.50$, $p < 0.001$). Leukocyte counts at baseline were associated with HIV-1 RNA and sTNFR II at baseline ($r = -0.36$, $p < 0.05$). The change in HIV-1 RNA levels, in CD4⁺ T cell counts and the change in immune activation markers did not correlate with the change in leukocytes, granulocytes and thrombocytes.

A direct influence of HIV in the development of leukopenia, granulopenia and lymphopenia during HIV-1 infection is unlikely. In the development of HIV associated thrombopenia not general immune activation but HIV itself seems to play a pathogenic role. Increase in leukocyte, granulocyte, lymphocyte and thrombocyte counts during antiretroviral therapy is not associated with decrease in general immune activation.

Neopterin - IFN- γ and their role during immunosenescence

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An increased production of pro-inflammatory cytokines and neopterin are characteristic features of old age. As influenza vaccination is not accompanied by the production of specific antibodies in a high percentage of elderly persons, we analyzed the nature of the underlying defect by characterizing T cell subsets and their clonal composition as well as their in vitro responsiveness to influenza antigen in young (< 35 years) and old (> 60 years) volunteers. The old age group consisted of two subgroups, (a) "responders", persons who did and (b) "non-responders", persons who did not produce specific antibodies following influenza vaccination. Old age was in general associated with typical changes in the T cell repertoire. Decreased diversity due to the appearance of large expanded clones was accompanied by increased numbers of CD45RO⁺ and of CD45RA⁺ CD11a⁺⁺CD28⁻ T cells. These changes were more pronounced in old non-responders than in responders. CD4⁺ and CD8⁺ T cells from non-responders produced interferon-(IFN)- γ , but produced only little IL-5 following in vitro stimulation with PHA or influenza antigen. In contrast, stimulated T cells from young persons and from aged responders produced both cytokines in vitro. Serum neopterin concentrations were higher in old nonresponders than in responders and young persons indicating an increased body load of IFN- γ . Overproduction of IFN- γ leading to a loss of IL-5 production seems to be the basis for the failure to produce specific antibodies following influenza vaccination in the elderly.

Non-transferrin bound iron and parenteral iron preparations: in vitro iron donating properties and response of cultured cells

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It is widely assumed that the standard parenteral iron preparations are degraded in the reticuloendothelial cells and that the iron is subsequently incorporated into transferrin or that they donate iron directly to plasma transferrin. Liver parenchymal cells have been considered as not affected. We show here that this picture is incorrect. The in vitro binding of iron from parenteral iron preparations (iron saccharate (*Ferrum vitis*®, *Venofer*®), iron gluconate (*Ferrlecit*®), iron dextran (*INFeD*®)) and ferric ammonium citrate (FAC) to isolated apotransferrin or normal human serum was assayed by separating the apo-, monoferric- and diferric transferrins on 6M urea polyacrylamide gel electrophoresis and immunoblotting. Primary cultures of rat hepatocytes and HepG2-cells were first depleted of iron by treatment with 50 μ M desferrioxamine overnight and then incubated with the iron preparations. Uptake of non-transferrin bound iron (NTBI) was measured as uptake from ferric DTPA. IRP activity in cytosolic extracts was analysed by RNA-bandshifts using as probe the human ferritin H chain transcribed from the pSPT-fer plasmid. Iron content of the cells was assayed by the permanganate-ferrozine-method. Expression of DMT-1 protein, and ferritin was assayed by immunofluorescence and by Western blots. Due to the polymeric nature of the iron complexes, colorimetric methods commonly used for assessing the serum iron content do not completely release the metal from the parenteral iron preparations and therefore yield too low values. A drastic digestion of the sample with permanganate in hydrochloric acid was necessary to set all iron free for the colorimetric reaction. Moreover the conventional method to estimate the serum iron-binding capacity by calculating the transferrin saturation with the serum iron concentration can be misleading after intravenous iron administration. Using urea gel electrophoresis we show that in contrast to FAC, none of the parenteral iron preparations can donate iron to transferrin. Ferric saccharate as well as ferric gluconate donate iron to hepatocytes and HepG2-cells practically as efficient as FAC, whereas ferric dextran is completely inert. The accumulated iron cannot be released by treatment with desferrioxamine nor with apotransferrin. Iron loading leads to stimulation of NTBI uptake and to a significant inactivation of IRP binding activity. DMT-1 expression is strongly increased in iron loaded mouse fibroblasts and HepG2-cells.

There is no direct delivery of iron from the par-

enteral iron preparations to transferrin in buffer as well as in serum. Polymeric iron preparations (ferric saccharate and ferric gluconate, but not iron dextrane) can be accumulated in hepatocytes and they are metabolically active at concentrations exceeding 50µM, but not chelatable. Thus different pools of intracellular iron seem to cause iron stress in the cells represented by increased NTBI uptake rates. Thus, during iv. iron administration care should be taken to keep plasma concentrations of iron below 3mg/l to avoid untoward side effects.

Neopterin screening of blood donations - recent data

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In 1986 neopterin screening of blood donations was introduced as an additional screening test to improve the safety of blood transfusions. In 1994 neopterin screening became mandatory for all Austrian blood banks. The target of this study was to obtain special physical data of blood donors and to correlate them with their neopterin concentrations. 1345 consecutive Tyrolean blood donors were examined for body weight, body size, body temperature, blood pressure, hemoglobin concentration, cholesterol, ALT values and neopterin concentrations. Additionally they were asked for their smoking habits. Further data which were used for statistical evaluation were age, sex and body mass index (BMI) of the donors. We performed correlation analysis (Pearson) of the parameters. In a first analysis of the data we found statistically significant correlations between neopterin and body weight ($p = 0.04$), neopterin and BMI ($p = 0.008$), neopterin and age ($p < 0.001$), neopterin and hemoglobin ($p = 0.04$). Controlling the correlations for age, none of the correlations remained significant. The results show that there is a highly significant statistical correlation between age and neopterin values. This fact was reported also previously by other authors. We could not find any confirmation for correlations between neopterin values and other physical conditions in the examined donor cohort. All the at first suspected correlations between neopterin and other parameters turned out to be age dependent.

Immunological changes during different types of prolonged exercise

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Several studies focused on the stimulation of the immune system during and after prolonged strenuous exercise. However, data are still conflicting possibly due to the different types and duration of exercise and the environment per se (e.g. altitude). In order to investigate these contributing factors several different exercise studies were performed: *Study A*: Oetzal bicycle marathon (OEM), 230 km, vertical displacement 5000 m, $N=8$; *Study B*: Swiss Alpine Marathon (SAD), 67 km, vertical displacement 2300 m, $N=13$; *Study C*: Berlin Marathon (BM), 42,192 km, $N=15$; *Study D*: AMAS 2000, 3-weeks stay at 1700 m (Oberlech, Austria) of patients with the metabolic syndrome (hypertension, obesity, dyslipidemia, insulin resistance) performing typical mountain holidays (walking tours 4 times /week, 3-4h). Measured serum parameters were TNF- α , TNF-R1, TNFR-II, IL-6, neopterin. For statistical evaluation two way analysis of variance (Friedmann) and Wilcoxon signed rank test were employed.

IL-6 was increased immediately after exercise termination at the OEM and SAD. TNF- α was unchanged after the OEM but slightly elevated within the first 5 days after the SAD. This increase in TNF- α was accompanied by increased concentrations of both TNF-Rs. Proinflammatory cytokines remained unchanged during the AMAS-study. Neopterin was significantly elevated immediately after the OEM and the SAD and still remained elevated for the first 72h. In contrast, no change in neopterin was found after the BM. During the AMAS-study neopterin was elevated on day 4 and day 18.

Different types of strenuous exercise seem to exhibit an alteration in immune activation. Physical exercise under moderate hypoxic conditions may activate the cellular immune system, as evidenced by elevated neopterin concentrations after the OEM and SAD. However, the absolute increase was small, still remaining below 10 nmol/l in 2/3 of the participants.

Modulatory effect of selective serotonin reuptake inhibitors on the human myelomonocytic cell line THP-1

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In recent studies of depressive patients different immunological alterations have been reported. Although the results are controversial there might be evidence for an immune activation in certain states of depression. Immunological alterations due to antidepressive medication has also been addressed in several studies most of them investigating the influence of tricyclic compounds or MAO-inhibitors. In contrary little is known about possible immune modulatory effects due to selective serotonin reuptake inhibitors (SSRI's). The aim of the present study is to evaluate possible immune modulatory effects caused by the SSRI compound citalopram by using THP-1 cell cultures. Effects induced by citalopram in the presence and absence of interferon- γ (IFN- γ) were determined by measuring neopterin, tryptophan and kynurenine as well as the kynurenine/tryptophan ratio. THP-1 liquid cell cultures were incubated with increasing doses of citalopram (0, 20, 100, 1000 mg). Furthermore a comparison between cultures stimulated with and without 100 U/ml IFN- γ was carried out. The immune parameters were evaluated after 24, 48 and 72 hours of incubation.

The most characteristic results were found after 48 hours of incubation. In cultures without IFN- γ stimulation no detectable neopterin levels were found. A dose of 20 mg citalopram did not alter tryptophan and kynurenine levels. A dose dependent increase of tryptophan and a decrease of kynurenine as well as a decrease of the kynurenine/tryptophan ratio was detected after an incubation with 100 and 1000 mg citalopram. Cultures stimulated with IFN- γ showed a totally different picture: Neopterin, kynurenine and the ratio were significantly increased whereas tryptophan was significantly decreased. This was found for 20 and 100 mg citalopram; with 1000 mg citalopram all measured parameters did not differ from control values.

Although these results can only be considered preliminary and further experiments are necessary one could speculate that in cell cultures without IFN- γ stimulation citalopram induces a suppression of the IDO enzyme-activity whereas in cultures with IFN- γ stimulation a cytotoxic effect might be seen which is

possibly enhanced by citalopram.

Cerebrospinal fluid neopterin and vasospasm in patients with subarachnoid hemorrhage

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Subarachnoid hemorrhage (SAH) with an incidence of approximately 15 per 100 000 population represents about 5-10 % of all non-traumatic intracranial hemorrhages. The most common cause of SAH in adults is the rupture of an aneurysm. SAH has a dismal prognosis. The course of the patients surviving the initial insult is further complicated by the malignant triad of hydrocephalus, rebleeding and cerebral vasospasm (CVS). CVS is diagnosed in 70 % of all patients and exceeds the morbidity and mortality of rebleeding and other complications. Therefore it poses a tremendous unsolved problem in the postoperative management of SAH after aneurysm rupture. The genesis of CVS is still not clear. In this study we pursued the idea that an aseptic subarachnoid inflammation triggers CVS. With a reliable parameter reflecting this rising intracranial inflammation it would be possible to predict the development of CVS. In such patients the CVS therapy could be started before CVS evolves or becomes symptomatic.

From February 2000 until February 2001 13 patients were included in this study. Only patients who received a ventricular drainage to treat intracranial pressure (ICP) elevation were included in my study because so CFS samples could be obtained non invasive and without any risk and discomfort for the patient. The median age of the patient population was 61.8 (44 - 79 years), 7 were females (58.3%). As control group served the patients who did not develop any cerebral vasospasm within the days of observation. All of the patients presented with normal renal function. Measurement of neopterin was performed in serum and cerebrospinal fluid (CSF) of the patients with the intention to determine a possible inflammatory reaction to the blood clot in the subarachnoid space. Increased amounts of neopterin are produced from human monocytes/macrophages and neopterin concentrations in body fluids are known to reflect both active cellular immunity and oxidative stress. Among others, these two mechanisms could lead to metabolic impairment, cellular disruption even necrosis, blood brain

barrier dysfunction and intracellular raise of Ca^{2+} concentration all resulting in muscular concentration and rigor like state presenting in the clinical picture as early onset or delay cerebral vasospasm.

We found an increase of intrathecal neopterin concentration after SAH which confirms earlier observations. The comparison between neopterin and C-reactive protein (CRP) in CSF and serum showed that this rise of neopterin is not secondary to an overall septic complication. Neopterin concentrations in serum and moreover in CSF depicted more precisely the ongoing intracranial aseptic inflammation than CRP did. We gained the impression of a CSF neopterin peak on day 3 and 4 and of a higher CSF CRP concentration in CSV patients, but this study could not demonstrate an association between CSF neopterin concentrations and CVS development in the first 7 days after SAH. In another study a correlation between these two parameters was demonstrated in the later course, however, the size of our population is still too low for a definite conclusion.

Mechanisms for the regulation of duodenal metal transporter-expression by iron

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Intestinal iron absorption is regulated by the body's demands for iron. Identification of divalent metal transporter 1 (DMT1) and ferroportin1 (FP1) has improved our understanding of iron transport across the intestinal epithelium. Although, DMT-1 and FP1 mRNA bear an iron responsive element (IRE) within its untranslated regions which should cause susceptibility to iron mediated regulation the latter has not been shown so far. Methods: The effects of iron perturbations on DMT-1 and FP-1 expression were investigated in CaCo2 cells and in primary tissue cultures of human duodenal biopsies by means of Northern Blot, Western Blot and Immunofluorescence, RNA-band-shift and Nuclear Run off analysis. Results: Both, DMT1 and FP1 mRNA levels were increased upon treatment of CaCo2 cells with desferrioxamine, whereas iron treatment resulted in the opposite effect. These changes were paralleled by the respective alterations in DMT-1 and FP1 protein expression. Although desferrioxamine increased the affinity of iron regulatory protein-1 to DMT-1-IRE, the mRNA half life of DMT-1 mRNA remained unchanged. Nuclear run-off analysis then demonstrated that the effects of iron and desfer-

rioxamine on DMT-1 and FP-1 mRNA expression are due to modulation of the transcription of these genes. Conclusion: Our results demonstrate that iron regulates the expression of the two ferrous ion transporters DMT-1 and FP-1 by affecting their transcription. This provides evidence for a negative feed-back loop between intracellular iron availability and transmembrane iron transport.

GTP cyclohydrolase I of *Physarum polycephalum*: genomic structure, mRNA variants and expression during cell cycle and differentiation

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Physarum polycephalum, an acellular or true slime mold, is one of few species identified which is capable of synthesizing both 5,6,7,8-tetrahydrobiopterin and folate. Here, we characterized GTP cyclohydrolase I, the key enzyme of this biosynthetic pathway, from *Physarum*. During differentiation into spherules as well as during achieving sporulation competence, two processes induced in vitro by starvation, GTP cyclohydrolase I expression is down-regulated whereas it remains unchanged during the naturally synchronous cell cycle. From a cDNA library, we isolated two GTP cyclohydrolase I clones. One encoded a 228 amino acid (25.7 kDa) type 1 protein which was functionally active upon recombinant expression, the other encoded a 195 amino acid (22.1 kDa) type 2 protein differing from the 228 amino acid protein at the C-terminus. This shorter protein was inactive. Sequence analysis showed that the type 2 mRNA is a splice variant of the type 1 mRNA and that splicing occurred at a position homologous to the boundary of exons 5 and 6 from human GTP cyclohydrolase I. Three of four splice variants identified so far for human GTP cyclohydrolase I are alternatively spliced at this position. In order to see whether this homologous position is an exon/intron boundary in *Physarum* too, we characterized the genomic structure of *Physarum* GTP cyclohydrolase I by PCR. We found, that the *Physarum* GTP cyclohydrolase I gene consists of 7 exons as compared to 6 exons for man and mouse, 5 exons for *Drosophila melanogaster*, 2 exons for *Dictyostelium discoideum*, and 1 exon for *Saccharomyces cerevisiae*. Alternative splicing of *Physarum* GTP cyclohydrolase I mRNA occurs also at the 3'-most exon boundary which is in

this case the boundary between exons 6 and 7. Not only the position of *Physarum* intron 6 but also that of intron 3 is homologous to human GTP cyclohydrolase I. In addition, the position of *Physarum* GTP cyclohydrolase I intron 1 is conserved in *Drosophila* and *Physarum* intron 4 is at a homologous position to the only *Dictyostelium* intron. *Physarum* introns 2 and 5 have no corresponding introns in the other species studied so far. Comparing protein sequences, the highest degree of homology of *Physarum* GTP cyclohydrolase I is found with *Dictyostelium*, a cellular slime mold. Genomic organization, however, places *Physarum* much closer to higher eukaryotes than *Dictyostelium*. The function of alternatively spliced GTP cyclohydrolase I proteins still remains to be elucidated. Provided that these proteins are expressed intracellularly they potentially could participate in formation of decameric GTP cyclohydrolase I and thereby affect enzyme activity. Interestingly, in different human cell types, quantification revealed that splice variants constitute up to 20 % of GTP cyclohydrolase I mRNA. Future work will clarify whether this comparatively high amount is caused by erroneous splicing or whether it represents a directed process and a means of regulation.

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Tetrahydrobiopterin concentrations and nitric oxide synthase activity in *Octopus vulgaris* Cuvier 1797

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Nitric oxide synthase inhibitors are known to block touch learning and visual learning in *Octopus vulgaris* (Robertson JD, Bonaventura J, Kohm A, Hiscat M, Proc R Soc Lond B Biol Sci 1996, 263(1377):1739-43). Presence of nitric oxide synthase activity and tetrahydrobiopterin, however, had not yet been demonstrated in this species. We therefore studied distribu-

tion of nitric oxide synthase activity and of tetrahydrobiopterin concentrations in the following tissues of eight specimens of *Octopus vulgaris*: brain (sub and supra esophageal ganglia), optic lobe, eye glands, brachial nerves, stellate ganglia, heart, gill heart, gill, blood, liver (testinal gland), ink gland, kidney (emuntories), muscle, skin, gonads, and, in case of females, oviduct glands. Tetrahydrobiopterin levels were estimated by HPLC with fluorescence detection following acidic and alkaline oxidation. Nitric oxide synthase activity was measured in presence of 3 μ M free Ca^{2+} by quantification of the formation of [^3H] citrulline from [^3H] L-arginine. Values were related to total protein in cell extracts as measured by the Bradford assay using bovine serum albumin as a standard.

Tetrahydrobiopterin and nitric oxide synthase activity were detected in all tissues, except for the blood, which contained only tetrahydrobiopterin but no nitric oxide synthase activity. Highest nitric oxide synthase activity was found in muscles (175 pmol/(mg.min)) and liver (34 pmol/(mg.min)), lowest levels were observed in gill heart (1.8 pmol/(mg.min)) and optic lobes (2.0 pmol/(mg.min)). Tetrahydrobiopterin levels did not follow this pattern: Highest levels were found in gonads (229 pmol/mg), followed by liver, kidney and eye glands (80 pmol/mg). Lowest levels were observed in muscles (5.4 pmol/mg) and gill heart (2.8 pmol/mg). These results demonstrate that nitric oxide synthase and tetrahydrobiopterin are widespread throughout the body of *Octopus vulgaris*. The lack of correlation of tetrahydrobiopterin concentrations and nitric oxide synthase activities suggests that, like in mammals, other tetrahydrobiopterin-dependent enzymes like e.g. phenylalanine hydroxylase may require the highest concentrations of the tetrahydrobiopterin cofactor in these tissues.

Comparison of urinary neopterin concentrations in individuals with household contact

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Neopterin concentrations in serum and urine are known to sensitively reflect activation of cell-mediated immunity. In this study, urinary neopterin concentrations of persons with household contact were determined over a one year period and the course of neopterin levels was compared. A family consisting of

1 child (aged 5 years), her mother and father, and her grandmother and grandfather was tested. All 5 individuals lived in the same house, child and her parents separated by one floor from the grandparents. Urinary samples were taken every second week during 1 year beginning in June. Neopterin concentrations were determined by high performance liquid chromatography (HPLC). In the child, neopterin concentrations were stable during the first 3 months, remaining far below 500 $\mu\text{mol/mol}$ creatinine. In contrast, during the subsequent 9 months 5 episodes with remarkable elevations of neopterin concentrations were detected in the child right after it started going to kindergarten for her second year; the 5 peak increases (middle of October, end of December, begin of February, begin of March and middle of May) reached levels between 850 and 1950 $\mu\text{mol/mol}$ creatinine. These peaks were associated with mild or moderate signs of infectious disease (fever, headache, or diarrhea) but infections were not serologically confirmed and did not belong to classical infectious diseases in children, e.g., measles, rubella or varicella infections. Comparing the course of neopterin concentrations between the persons tested, we detected several corresponding elevations of urinary neopterin in the child and her relatives. The best association was detected between the child and her father (3/3 peaks of neopterin concentrations correlated with the peaks in the child and reaching levels of 300 to 450 $\mu\text{mol/mol}$ creatinine whereas all the other values remained below the upper limit of normal of 182 $\mu\text{mol/mol}$ creatinine) and between the child and her mother (2/2 corresponding neopterin peaks). One highly increased urinary neopterin concentration was observed in the grandmother (720 $\mu\text{mol/mol}$ creatinine, upper limit of normal: 249 $\mu\text{mol/mol}$ creatinine) which corresponded to a peak in the child, a similar observation was made between the child and her grandfather. In fact, there were only 2 peaks in the child's relatives exceeding the upper limit of normal which appeared to be independent from a corresponding increase in the child. In summary, a high correspondence in neopterin peaks was detected between child and adults and they seemed to be associated with infections. The data render it likely that the child was transmitting infectious diseases which she acquired at the kindergarten to her relatives. It also appeared that her parents were more often challenged by infections than the grandparents were. The data are in line with the assumption that a closer family contact between individuals and persons is increasing the risk of disease transmission via household contact. Further studies have to be performed to confirm these suggestions.

Immunomodulatory effect of aqueous extracts of Vietnamese *Crinum latifolium* (L.) and other teas in human peripheral blood mononuclear cells

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In Vietnamese and Chinese traditional medicine hot aqueous extract of *Crinum latifolium* is used because of its antitumor activity. The genus *Crinum* is thought to possess antiviral and immunostimulative properties. Similar qualities were observed in green and black tea derived from *Camellia sinensis* (L.). A growing body of evidence suggests that moderate consumption of green and black tea may protect, e.g., against several forms of cancer, cardiovascular diseases, and bacterial infections. In this study, the immunomodulatory property of *Crinum latifolium* (L.) extracts should further be investigated and compared to those of black and green tea. Human peripheral mononuclear cells were cultured in the presence of tea extracts with or without mitogens or interferon- γ . The effect of plant extracts on cultured cells was assayed by neopterin production, a sensitive marker reflecting the activation of cell-mediated immunity. Our experiments show that extracts of *Crinum latifolium* (L.) slightly enhance neopterin production in unstimulated peripheral mononuclear cells, whereas an effective reduction of neopterin formation in cells stimulated with concanavalin A, phytohemagglutinin, or interferon- γ was observed. Green and black tea extracts displayed similar immunomodulatory properties in our in vitro system, whereas *Crinum latifolium* (L.) extracts seemed to be more effective in reducing neopterin formation in stimulated cells.

Osteoprotegerin levels in kidney allograft recipients

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Osteoprotegerin (OPG) is a soluble decoy receptor blocking the interaction between RANK (Receptor activator of NF- κ B, expressed on osteoclasts and their precursor cells), and its ligand RANKL. Thus osteoclastogenesis and osteoclast activation is attenuated by OPG. We have developed a sandwich immunoassay suitably sensitive to measure circulating levels of OPG in serum. Reference levels established for a group of healthy persons (age 19 - 74 years, median 38 years) were 0.05 (limit of detection) - 0.145 ng/ml (5th - 95th percentile) with a median value of 0.043 ng/ml.

In patients after kidney transplantation, OPG levels were between 0.05 and 1.20 (0.05 - 0.98 ng/ml as 5th - 95th percentile; median 0.11 ng/ml). These values are significantly above the reference range. However, there were no correlations with either the age of the patients, their serum calcium, the immunosuppressive therapy (Cyclosporin and/or steroids) or kidney function. We have to conclude that the OPG regulatory system is independent of the function of the grafted kidney, and not influenced by the dose of steroids. This is in contrast to studies on osteoblasts in vitro, where it was shown that corticosteroids are the most potent inhibitors of OPG synthesis.

In patients on chronic hemodialysis, OPG levels ranged from 0.02 to 0.45 ng/ml (median 0.13 ng/ml). There was a negative correlation to the vitamin D status (25OHD and 1,25(OH)₂D). Furthermore, OPG levels were relatively lower in patients with non-renal osteopathy. Analysis of OPG and the circulating ligand sRANKL will be necessary to evaluate the contribution of these factors to the pathological changes in renal insufficiency and after transplantation.

Degradation of tryptophan in HIV-infected patients treated with HAART

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In vitro, interferon- γ activates the enzyme indoleamine (2,3)-dioxygenase (IDO) in various cells and cell lines. This enzyme degrades tryptophan via the kynurenine pathway. Recently it has been demon-

strated that IDO activation in monocytes/macrophages is associated with T-cell unresponsiveness in vitro and that IDO activation is crucial for the survival of the fetus in pregnant mice. In HIV infected individuals we have demonstrated an enhanced degradation of tryptophan already some years ago. In light of the recent data, we further examined the rate of tryptophan degradation (expressed as the kynurenine per tryptophan ratio; kyn/trp ratio) in patients with HIV infection under highly active antiretroviral treatment (HAART). We evaluated 71 treatment naive patients during the first six months of HAART for the change in levels of tryptophan, kynurenine and the kyn/trp ratio, and we compared the data with earlier results on HIV-1 RNA, the change in the CD4⁺ T-lymphocyte count, and serum neopterin. Patients were either treated with two nucleoside analogues and one non-nucleoside analogue reverse transcriptase inhibitor or received antiretroviral combination therapy including protease inhibitors. Before therapy, kynurenine and the kyn/trp ratio correlated with virus load (kynurenine $r_s = 0.378$, $p = 0.001$; kyn/trp ratio 0.328 , $p = 0.005$) and neopterin levels (kynurenine $r_s = 0.426$, $p < 0.001$; kyn/trp ratio 0.579 , $p < 0.001$) but not with CD4⁺ cells counts. Tryptophan concentrations correlated significantly only with neopterin ($r_s = -0.366$; $p < 0.001$). During therapy, average tryptophan levels increased from 44.5 μ M at initiation of therapy to 54.9 μ M six months later ($p < 0.001$), in the same time kynurenine (3.05 vs. 2.63 μ M; $p = 0.001$) and kyn/trp ratios (76.7 vs. 48.7 mmol/mol; $p < 0.001$) decreased. Changes of tryptophan and of the kyn/trp ratio during follow up were significantly associated with changes of neopterin (tryptophan $r_s = 0.277$, $p < 0.05$; kyn/trp ratio: 0.359 , $p = 0.01$). Close associations were found of tryptophan metabolic changes with HIV load and neopterin concentrations but not with CD4⁺ cell counts which agrees with the concept that interferon-gamma is the common background of IDO activation and of neopterin formation. Our data do not support a role of IDO activation in the development of CD4⁺ cells deficiency, rather they confirm the close relationship of chronic immune stimulation with HIV reproduction.

Changes of hemoglobin levels in patients treated with antiretroviral therapy

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Anemia is a frequent complication in the course of HIV infection. Lower hemoglobin levels were found to be significantly associated with accelerated progression to AIDS and death. In vitro, HIV is able to infect erythroid progenitor cells. In humans with HIV infection and in monkeys with experimental SIV infection virus could not be detected in isolated progenitor cells. In HIV-1 infected patients lower levels of hemoglobin are associated with increased levels of serum and urinary neopterin. Highly active antiretroviral treatment (HAART) is associated with a decrease in immune activation, including decreased levels of neopterin.

In this study we addressed the question whether the decrease of immune activation in antiretroviral treated patients is associated with an increase of hemoglobin levels? To answer this question we evaluated consecutively recruited, treatment naive patients during the first six months of HAART for the change in levels of immune activation markers (neopterin, sTNFR II), CD4⁺ T cells, HIV-1 RNA and hemoglobin. Women and patients having received therapy for anemia were excluded from this retrospective study. The study population comprised 35 individuals, mean age 39.0 ± 10.1 years, 22 had AIDS. The median hemoglobin levels increased from 13.9 g/dl at initiation of therapy to 14.9 g/dl six months later ($p < 0.001$) and median CD4⁺ T lymphocytes increased from 101/μl to 227/μl ($p < 0.001$). Median HIV-1 RNA levels decreased from 5.38 log₁₀ copies/ml to 2.22 log₁₀ copies/ml and serum neopterin levels decreased from 20 to 7.8 nmol/l (for both, $p < 0.001$). At baseline hemoglobin levels correlated with CD4⁺ T cells ($r = 0.33$, $p < 0.05$), HIV RNA ($r = -0.41$, $p < 0.01$) and much stronger with plasma neopterin ($r = -0.64$, $p < 0.001$). A correlation was found between the change in hemoglobin levels and the change in neopterin levels ($r = -0.39$, $p = 0.02$). The change in HIV-1 RNA levels and the change in CD4⁺ T lymphocytes did not correlate with the change in hemoglobin levels ($r < 0.3$, $p > 0.1$).

Although correlations among pathologies cannot be considered to be equivalent to causation, our data are consistent with the view that HIV may induce chronic immune stimulation and concomitant release of inhibitory cytokines (e.g. interferon- γ and TNF- α) which in turn are responsible for inhibition of erythropoiesis.

Expression of the duodenal iron transporters divalent metal transporter1 and ferroportin1 in iron deficiency and iron overload

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Intestinal iron absorption of non heme iron is likely to be mediated by DMT1, a recently identified ion channel specific for divalent metals such as Fe²⁺, Cu²⁺, Zn²⁺, Mn²⁺ and Pb²⁺. In rats the duodenal expression of DMT1 has been shown to be inversely related to dietary iron intake. Moreover, duodenal DMT1 expression is highly increased in patients with hereditary hemochromatosis. However, no information was so far available concerning duodenal DMT1 protein expression and localization in humans. Thus, we investigated in duodenal biopsies from patients with different pathologies of iron homeostasis the tissue distribution of DMT1 and its dependence on the body's iron demands. Immunohistochemistry, Western blot analysis and quantitative RT-PCR were performed in duodenal biopsies taken from a total of 60 patients with iron deficiency anemia, anemia of chronic diseases, secondary iron overload, and hereditary hemochromatosis and peptic ulcer disease. Immunohistochemistry showed a specific staining within the brush border membrane of duodenal epithelial cells, while hardly any DMT1 expression was observed in duodenal crypt cells. Differences were observed in the intensity of the staining between various types of iron metabolism disorders. As confirmed by Western blot analysis highest expression of DMT1 was seen in patients with hereditary hemochromatosis, whereas in biopsies taken from subjects with secondary iron overload only a weak staining could be observed. In iron deficiency anemia by means of quantitative RT-PCR an inverse correlation between duodenal DMT1 mRNA levels and serum ferritin levels could be seen. Regulation of iron absorption via DMT1 expression appears to be a central regulatory tool of the organism to adjust duodenal iron uptake to the body's iron demands. The central regulatory factor for DMT1 expression appears to be the intracellular iron content of enterocytes which may be low in hereditary hemochromatosis due to the HFE mutation, thus leading to extraordinary high expression of DMT1 in this disease.