

Pteridines  
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## Abstracts

# Twenty-sixth International Winter-Workshop on Clinical, Chemical, and Biochemical Aspects of Pteridines

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Organized by D.Fuchs (Innsbruck), G.Reibnegger (Graz), U.Köller (Vienna)

### **Current Data from the Project "Lipometry, Lifestyle and Cardiovascular Risk Parameters" at the Styrian National Exhibition 2006 "Ways to Health"**

Almer A, Lang N, Nemet A, Dolinar U, Sargsyan K, Horejsi R, Möller R, Truschnig M, Mangge H

Clinical Institute for Medical and Chemical Laboratory Diagnosis, and Institute for Physiological Chemistry, Medical University of Graz, Austria

Epidemiologic studies showed high rates of obesity (especially males) in Styria as compared to other Austrian provinces such as the Tyrol. To investigate the current state of public health in Styria, a project called "Lipometry and lifestyle" was started as an essential part of the program of the national exhibition "Ways to Health 2006" in Bruck and der Mur.

Lipometry based on an optical device, which enables a non-invasive, quick, safe and precise measurement of the subcutaneous adipose tissue (SAT) thickness was offered to all visitors. Additionally, a clinical investigation (age, height, weight, Body mass index, history of myocardial infarction etc.), and measurements of total cholesterol, HDL-cholesterol, glucose, pulse rate and blood pressure was performed. Based on these data, individual risk profiles were discussed with the visitors. All variables were electronically stored in a SPSS data base.

Within 6 months 5000 probands were enrolled in the study. The cohort showed an average age-

but not an average gender-distribution. The male participants were more afflicted with overweight (BMI between 25 and 30kg/m<sup>2</sup>) and showed a significantly higher percentage of visceral adipose tissue (VAT) compared to females. The rate of obesity (BMI >30kg/m<sup>2</sup>) was equal for both genders. The females showed higher HDL-cholesterol and lower systolic and diastolic blood pressure levels throughout all investigated age quartiles. As compared to body mass index (BMI), waist circumference, waist/hip-ratio, the waist/height-ratio (w/htr) turned out to be the best risk predictor for increased systolic blood pressure, cholesterol, glucose and critically decreased HDL cholesterol irrespective of sex, age and BMI values. A w/htr of 0.5 was found to be the critical upper limit.

In conclusions, for an individual and simple risk prediction, people should take care of their waist to keep it well below half their height.

### **IFN- $\gamma$ -mediated Pathways in Patients with Fatigue and Chronic EBV-Infection**

Bellmann-Weiler R, Schroecksnadel K, Holzer C, Fuchs D, Weiss G

Department of General Internal Medicine, Clinical Immunology and Infectious Diseases and Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck, Austria

Chronic active EBV infection is characterized by mononucleosis like symptoms such as fatigue,

lymphadenopathy and/or hepatosplenomegaly together with serologic evidence for ongoing EBV replication. Expansion of cytotoxic T- cells and a T helper cell type -1 mediated immune response driven by the production of interferon-gamma (IFN- $\gamma$ ) are essential for controlling EBV infection. IFN- $\gamma$  triggers several antiproliferative and antiviral mechanisms in target cells as there are the induction of nitric oxide synthase, the production of reactive oxygen species or indoleamine (2,3-dioxygenase (IDO)). Thereby, IDO degrades essential amino acid tryptophan to restrict protein biosynthesis. Because tryptophan is also precursor of neurotransmitter 5-hydroxytryptamine (serotonin), tryptophan depletion due to the induction of IDO can lead to mood disturbances and impaired quality of life in patients suffering from diseases involving chronic cellular immune activation.

This study investigated the immune activation status as measured by neopterin concentrations and tryptophan metabolism in 20 patients with chronic active EBV-infection, who were followed up for 4 to 8 months and in 10 healthy controls. The clinical suspicion of chronic active EBV infection was verified by the presence of anti-EBV-IgM or anti-EA antibodies and/or upon detection of EBV DNA in peripheral blood using quantitative RT-PCR. Patients with detectable EBV-DNA in peripheral blood had higher serum neopterin ( $p < 0.01$ ) and lower tryptophan concentrations ( $p = 0.01$ ) than EBV-DNA negative patients. Serum neopterin concentrations, indicating Th-1 mediated immune activation, correlated with enhanced tryptophan degradation ( $r_s = -0.650$ ,  $p < 0.001$ ) in patients, but not in healthy individuals. Patients suffering from more severe symptoms (as assessed by questionnaires) tended to have enhanced tryptophan degradation.

In conclusion, our data show that EBV viremia is associated with cell mediated immune activation and increased tryptophan degradation, the latter may partly account for the fatigue found in these disorders. Further studies are needed to clarify a possible association between tryptophan depletion and the severity of fatigue in patients with chronic EBV infection.

### **Protein Patterns of Sporulation- and Starvation-stages of *Physarum polycephalum***

Bommassar C, Groebner P, Werner-Felmayer G, Werner ER, Golderer G

Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck, Austria

The real slime mold *Physarum polycephalum* is a eukaryote with a calcium independent NO synthesis to gain sporulation competence (1). After a 5 day starvation-period the plasmodia become competent and can be induced in the laboratory with light for sporulation. After light induction it takes about 14 hours to generate spores. To get more insight in the sporulation programme, we compared protein patterns in starved plasmodia with and without light pulse.

Plasmodia were grown on Petri dishes for 5 days. At 24, 72 and 120 hours plasmodia were scrape from the surface and stored by  $-80^{\circ}\text{C}$  until protein extraction. For sporulation samples we used a 30 minute far red ( $<700\text{ nm}$ ) light pulse for induction. At 3, 6, 9, 10.5, 11, 12.5 hours, cultures were divided in two equal halves. One was collected for analysis of protein; the other half was further incubated to verify that the cultures really sporulated. Control cultures were treated in exactly the same way, but the light pulse was omitted. Samples from five individual cultures were pooled for each time point, two such time courses were collected. For analysis of protein patterns we used the difference gel electrophoresis (DIGE) technique. Sample pairs to be compared were labelled with two different fluorescent dyes, mixed, and separated together by two dimensional gel electrophoresis. To allow for comparison of separate gels, an internal standard, labelled with a third different colour, was included in all separations. Gels were scanned for three different fluorescent colours by a Laser fluorescence scanner.

The threshold for expression difference was set to 2-fold, significance of difference was judged by Student's T-test. Starvation up-regulates 7 spots and down-regulates 72 spots out of a total of 1316 spots. The light pulse leads to up-regulation of 6 spots, and down regulation of 49 spots out of a total of 1628 spots at 12.5 hours. In earlier

sporulation (3h to 9h) no significant difference between induced and non induced plasmodia was found. From the sequence of these altered spots we hope to get a more comprehensive picture of proteins involved in the differentiation process of *Physarum polycephalum*.

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### **Elevation of the Immunomodulatory Enzyme Indoleamine 2,3-dioxygenase (IDO) in Tumor Cells Facilitates Immune Escape and Leads to Enhanced Metastases**

Brandacher G, Troester B, Kronberger IE, Fuchs D, Obexer P, Margreiter R, Amberger A

Department of General and Transplant Surgery, Tyrolean Cancer Research Institute; and Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Austria

Indoleamine 2,3-dioxygenase (IDO) is activated by IFN- $\gamma$  and via local tryptophan depletion modulates T-cell function and promotes immune tolerance. IDO has been demonstrated to be critically involved in maternal tolerance and recent attention has turned towards its role in immune evasion of certain tumors. However, if IDO expression is involved in tumorigenesis and tumor growth control still remains controversial.

Murine CT26 colon adenocarcinoma cells were transfected with the human IDO gene and stably expressing cells (CT26-IDO+ and CT26-vector control) were then injected subcutaneously into BALB/c mice. Macroscopic tumor growth was assessed after 12 days. Concentrations of kynurenine and tryptophan in culture supernatants and serum were analyzed by HPLC. Kynurenine to tryptophan ratio (kyn/trp) was calculated to estimate IDO-activity. IDO gene expression in cell lines and tumor tissue was assessed by quantitative PCR and Western blot analysis.

CT26-IDO+ cells exhibit high IDO enzyme

activity as determined by HPLC and expressed as the kynurenine to tryptophan ratio ( $\mu\text{mol}/\text{mmol}$ ) after 24 hours. *In vivo*, IDO activity (kyn/trp) determined in serum of tumor bearing mice was significantly higher ( $75.25 \pm 10.21 \mu\text{mol}/\text{mmol}$ ) in animals with CT26-IDO+ tumors as compared to  $37.29 \pm 7.85 \mu\text{mol}/\text{mmol}$  in animals with IDO negative tumors. All mice developed clinically evident tumors after six days, as confirmed by histology. Tumor size of CT26-IDO+ tumors was significantly smaller than that of CT26 vector controls ( $136.2 \pm 28.46 \text{ mm}^3$  vs.  $237.0 \pm 63.02 \text{ mm}^3$ ). However, only mice with CT26-IDO+ tumors developed peritoneal carcinosis, malignant ascites and distant metastases, whereas mice with CT26-IDO negative tumors showed locally restricted tumor growth.

In conclusion, this is the first preclinical model to demonstrate that IDO expression by colorectal tumor cells significantly contributes to disease progression, frequency of metastases and overall survival. Interfering with the IDO pathway by the use of IDO inhibitors might add a novel tool in the panel of cancer therapeutics and may enhance T cell-dependent antitumor immunity.

### **Exhaled Breath Analysis of Lung Cancer Patients: Preliminary Results**

Denz H, Bajtarevic A, Schmid A, Schwarz K, Kushch I, Fiegl M, Wolf F, Hilbe W, Amann A

Landeskrankenhaus Natters, Abteilung für Innere Medizin/Onkologie, Natters; Department for Anesthesiology and General Intensive Care, Department Therapeutic Radiology and Oncology, and Clinical Division for General Internal Medicine, Department for Internal Medicine, University Hospital Innsbruck, Innsbruck, Austria

The present study investigated volatile organic compounds (VOCs) in exhaled human breath for detecting lung cancer by proton-transfer-reaction mass spectrometry (PTR-MS). PTR-MS can be used without time-consuming preconcentration of the gas samples. Exhaled breath was collected in Tedlar bags and investigated by PTR-MS.

Concentrations of volatile organic compounds (VOCs) were obtained using a kinetic rate constant for protonation of  $k = 2 \cdot 10^{-9} \text{ cm}^3 \text{ sec}^{-1}$ . The concentrations are therefore uncalibrated.

VOCs leading to the product ion at  $m/z = 31$  (VOC-31, tentatively protonated formaldehyde),  $m/z = 43$  (VOC-43, tentatively a fragment of protonated iso-propanol), were found at significantly higher concentrations in the breath gas of lung cancer patients as compared with healthy volunteers. Formaldehyde is possibly a marker for endogenous immune activation (1) and therefore of special interest. VOCs leading to the product ion at  $m/z = 69$  (VOC-69, tentatively protonated isoprene) and the product ion at  $m/z = 63$  (tentatively protonated dimethylsulfide) were found at significantly lower concentrations in exhaled breath of lung cancer patients as compared with healthy volunteers. Decreased isoprene concentrations could be associated with low cholesterol levels (2) observed in cancer patients (3).

The results of this pilot study are preliminary. A detailed investigation of various factors, such as gender, age, indoor air contamination, breathing rate, pulse frequency and stage of cancer etc is necessary to understand and validate these results.

Within the limits of pilot study, VOC-31, VOC-43, VOC-69 and VOC-63 were found to discriminate between exhaled breath of lung cancer cases and healthy controls. The relatively simple and time-saving breath gas analysis by PTR-MS makes this method attractive for a larger clinical evaluation.

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cancer. *Int J Mol Med* 2000; 5:201-205

### **Protein Glycation Damage due to Chronic Glucose Exposure in Type II Diabetes**

Engin AB, Yetkin I, Fuchs D, Demirci H

Department of Endocrinology, Faculty of Medicine, University of Gazi, Ankara, Turkey, Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria

A dramatic endpoint of hyperglycemia is the generation of reactive oxygen species (ROS) leading to elevated oxidative stress that is a key factor in the pathogenesis of diabetic complications. Autoxidation of sugars and of the products of non-enzymatic glycation of proteins are closely linked to free radical-mediated reactions. A specific subtype of hemoglobin, HbA1c, indicates the possible level of glycation damage. Albumin is the major antioxidant proteins circulating in serum and continuously exposed to oxidative stress, while it can be modified into nonfunctional form by non-enzymatic glycation. ROS have been found to be related to tryptophan degradation and interfere with neopterin derivatives secreted by interferon- $\gamma$  stimulated macrophages. 82 healthy controls and 202 type II diabetes patients (Range: 0.1-33 years diabetes duration and Mean  $\pm$  SEM;  $190 \pm 4.9 \text{ mg/dl}$  fasting blood glucose (FBG)) were grouped according to the diabetes duration and FBG. FBG, serum albumin, uric acid, neopterin, tryptophan, kynurenine, hemoglobin, HbA1c were measured, and compared to the healthy controls and in between each subgroup. Compared to the controls, significant increase in HbA1c reached to a plateau when the diabetes duration was longer than 6 years. Serum albumin concentrations indicated a continuous and significant decrease from the second year of diabetes while neopterin levels were not increased ( $p < 0.05$ ). Serum albumin concentrations were significantly decreased in diabetic patients compared to the healthy controls. Categorizing the patients according to the FBG levels, HbA1c were significantly elevated when the FBG were increased. The patients with the FBG  $> 140 \text{ mg/dl}$

had lower serum albumin ( $p < 0.05$ ) and higher neopterin concentrations compared to the healthy controls ( $p < 0.05$ ). Uric acid levels did not affect by the increase of the FBG. No significant alteration was found in serum tryptophan and kynurenine levels of none of the diabetic patient groups and subgroups. In antioxidant defense, albumin that regards as the 50 % of total body protein, acts more predominantly than uric acid which is another antioxidant molecule. In diabetic patients macrophage stimulation and oxidative stress found to be related to increased diabetes duration and FBG.

### **A Sibling Case of DOPA Responsive Dystonia and Autism**

Fujioka H, Shintaku H, Yamano T

Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, 545-8585

Patient 1: A girl of 11 years old was admitted our hospital because of abnormal posture, and the decrease of the concentration of neopterin and biopterin in cerebrospinal fluid. She could not speak any word at 18 months old but could talk by three years old. She often tumbled down when she was running around at her age of 4 to 5. She often had odd posture around seven years old. At 10 years old, she was diagnosed as torsion dystonia. No diurnal fluctuation was observed. By administrating 10mg/kg/day of L-DOPA, her dystonic posture was improved. Oral dyskinesia was observed when the dose of L-DOPA was over 15 mg/kg/day. Oral administration of tetrahydrobiopterin and 5-hydroxytryptamine was added. After the treatment was started, high signal was detected in basal ganglia by T1 weighted MRI. Her parents were not related. Genetic analysis showed that DYT 1, GCH 1 and huntingtin were normal.

Patient 2: A nine years-old boy was a brother of patient 1. He could not speak any word at 18 months old. He was hyperkinetic. No dystonic posture, tremor nor akinesia has been observed. He can hear simple words now.

### **Antioxidant Enzyme and Major Pteridine Levels in Patients with Benign and Malignant Breast Tumors**

Girgin G, Sipahi H, Sahin TT, Yuksel O, Tekin E, Baydar T

Department of Toxicology, Faculty of Pharmacy, Hacettepe University; Department of Surgery, Faculty of Medicine, Gazi University; Ankara, Turkey

In mammalian cells, oxygen radicals are generated not only in mitochondria but also by neutrophils and macrophages as a part of immune response. Human tumor cell lines have been shown to produce large amounts of hydrogen peroxide *in vitro*. The antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) catalyze the transformation of peroxide and superoxide radicals to non-toxic species. In various studies two enzymes were shown to have increased expression or activity in breast tumor tissue compared to normal controls. Aromatic pterins like neopterin and biopterin do not directly interact with superoxide or peroxy radicals. Neopterin was suggested to have antioxidant effect not with its scavenging property but with indirect interactions. It is a sensitive marker for the activation of cellular immune system. Neopterin levels in body fluids can be regarded as an indirect estimate of the degree of oxidative stress during cell-mediated immune response. The amounts of neopterin produced by activated monocytes/macrophages correlate with their capacity to release reactive oxygen species (ROS). The aim of this study was to determine the levels of urine neopterin, biopterin levels and erythrocyte catalase and superoxide dismutase activities in patients having malignant ( $n = 23$ ) and benign ( $n = 28$ ) breast tumors. Mean neopterin level was  $158.8 \pm 92.34$   $\mu\text{mol/mol}$  creatinine in patients with malignant tumors of the breast and the biopterin levels were  $31.61 \pm 21.90$   $\mu\text{mol/mol}$  creatinine. In the patients with benign breast tumor, biopterin and neopterin levels were  $32.47 \pm 18.51$  and  $112.9 \pm 32.70$   $\mu\text{mol/mol}$  creatinine, respectively. There was a slight increase in neopterin levels in malignant patients but this difference was not statistical sig-

nificant. CAT and SOD activities were  $0.68 \pm 0.01$  U/mg protein to  $5.17 \pm 1.00$  U/mg protein in breast cancer patients, respectively. CAT and SOD activities of benign breast tumor patients were  $0.70 \pm 0.01$  U/mg protein and  $5.40 \pm 1.51$  U/mg protein, respectively. There was no difference between malignant and benign breast tumor patients in either CAT or SOD activities.

### **Renal Uptake of BH<sub>4</sub>: a Major Determinant of BH<sub>4</sub> Levels in the Body**

Hasegawa H, Sawabe K, Suetake Y, Maruyama S, Fujii R, Saeki Y

Department of Biosciences and Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, 409-0193 Japan

BH<sub>4</sub> levels in the body are maintained stably around age-matched standard which is rather lower in aged animals. Tetrahydrobiopterin (BH<sub>4</sub>) is synthesized *de novo* from GTP. Simultaneously, animals excrete a large amount of BH<sub>4</sub> daily in the urine. In order to maintain the steady state levels of BH<sub>4</sub>, various mechanisms regarding *de novo* biosynthesis, retrieval through salvage pathway, and excretion out to urine, might be finely tuned in the body. None-the-less, many neural or vascular disorders were reported in relation to inadequate BH<sub>4</sub> levels in the tissue. In case of BH<sub>4</sub>-supplementation therapy, the retention of incorporated BH<sub>4</sub> in quantity is not appreciable. In animal experiments, for example, tissue BH<sub>4</sub> levels rose greatly after administration of BH<sub>4</sub> but they returned to the previous levels in most organs within several hours. It was reported that a half the injected BH<sub>4</sub> (i.p.) was excreted in the urine within 24 hours. In this context, the urinary excretion of BH<sub>4</sub> might be one of the major determinant of BH<sub>4</sub> levels in the body. But the mechanism of BH<sub>4</sub> excretion has not been explored yet. In case of many bioactive materials and nutrients, plasma contents of small molecule are generally filtered out of glomerulus into primary urine, then selected materials are retrieved by absorption through epithelial cells of nephron.

We first paid attention on the dynamic partition

of BH<sub>4</sub> between the plasma and blood cells. We found that the plasma and blood cells were always exchanging BH<sub>4</sub> in running blood. It was noted that a fraction of BH<sub>4</sub> in the running plasma had been removed while red blood cells maintain enough BH<sub>4</sub> ready to supply it to the surrounding plasma. Second, we focused on whether kidney takes up BH<sub>4</sub> from urinary filtrates as was the case of metabolizable sugars and nucleobases. We got evidences which indicated that the kidney took up BH<sub>4</sub> from the primary urine. Hence the rest of BH<sub>4</sub> was to be discarded into urine. Third, we tried to attenuate the renal uptake of BH<sub>4</sub> with reagents known as inhibitor of membrane transporters. Within tested, benzbromarone, an anti-gout drug, inhibited BH<sub>4</sub> uptake by a concentration dependent manner: suggesting that the uptake process was driven, at least in part, by benzbromarone-sensitive transporter(s). The transporter system involved in BH<sub>4</sub> uptake is under investigation.

### **Cacao Extracts Suppress Tryptophan Degradation in Mitogen Stimulated PBMC**

Jenny M, Fischer B, Ledochowski M, Schennach H, Fuchs D

Division of Biological Chemistry, Biocenter, and Department of Internal Medicine, Innsbruck Medical University; and Central Institute of Blood Transfusion and Immunology, University Hospital Innsbruck, Innsbruck, Austria

Cacao refers to cocoa powder derived from the beans of *Theobroma cacao* by grinding and removing the cocoa butter from the dark, bitter cocoa solids. Polyphenolic cocoa compounds such as flavonols are thought to have a protective effect on cardiovascular health, cancer and diseases associated with inflammation and to improve impaired immune function.

In this study, the impact of commercially available pure cacao powder extracted in either water or ethanol (30%) on degradation of tryptophan and formation of neopterin was examined in freshly isolated human peripheral blood mononuclear cells (PBMC) *in vitro*. Within activation of

the cellular immune system by phytohaemagglutinin A (PHA), Th1-type cytokine interferon- $\gamma$  (IFN- $\gamma$ ) induces the enzyme indoleamine 2,3-dioxygenase (IDO) which converts tryptophan to N-formylkynurenine, which subsequently is deformed to kynurenine. To estimate the activity of the IDO enzyme, kynurenine and tryptophan concentrations were measured in the supernatants of PBMC by HPLC via fluorescence and UV absorption detection, the ratio of kynurenine and tryptophan concentrations was calculated as an index of IDO activity. In the same experiments the amount of neopterin released by the cells was determined via ELISA (BRAHMS Diagnostica, Hennigsdorf/Berlin, Germany), as another marker for the activation of T-cells and macrophages.

The tested cacao extracts significantly suppressed tryptophan degradation and neopterin formation in mitogen-induced PBMC in a dose-dependent manner, PHA-induced IDO activity was completely inhibited by addition of 5 $\mu$ g/ml ethanolic and aqueous cacao extracts. The inhibition of tryptophan degradation and neopterin production in parallel directs to a suppressive effect of the active compounds in cacao on activated T-cells and most notably, on production of IFN- $\gamma$ .

With regard to the well known effects of cacao products like chocolate on mood, bearing the capacity to lift spirits, to create highs and make people feel good and the well known implication of tryptophan metabolism in psychiatric diseases, the influence of the cacao extracts to suppress mitogen-induced IDO activation and tryptophan degradation suggests a possible connection of cacao compounds to the serotonergic system. On the basis of our results, we propose a new mechanism of mood elevation by cocoa and chocolate intake: cocoa-based products slow-down the activation of IDO and by this mode of action may enhance the availability of tryptophan for serotonin biosynthesis. In turn, cocoa intake may improve quality of life, especially in those patients suffering from inflammatory conditions.

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### **Influence of Non-peptidic $\delta$ -opioid Receptor Antagonists on *in vitro* Mitogen Stimulated Peripheral Blood Mononuclear Cells**

Jenny M, Winkler C, Spetea M, Schennach H, Schmidhammer H, Fuchs D

Division of Biological Chemistry, Biocenter, Innsbruck Medical University; Department of Pharmaceutical Chemistry, Institute of Pharmacy, Center for Molecular Biosciences, University of Innsbruck; and Central Institute of Blood Transfusion and Immunology, University Hospital Innsbruck, Innsbruck, Austria

Opioids are agents that interact with opioid receptors localized in the central and peripheral nervous system. However, opioid receptors are also expressed on various immunocompetent cells such as lymphocytes, monocytes and macrophages and were shown to alter immune function.  $\delta$ -Opioid receptor agonists were found to stimulate T and B cells and to activate granulocytes and monocytes. Conversely, immunostimulation can be blocked by the non-peptidic  $\delta$ -opioid antagonist natriindole (NTI). *In vitro*, NTI and other structurally related  $\delta$ -opioid antagonists inhibit allogeneic mixed lymphocyte reaction, natural killer cell activity and reduced interleukin-2 production in lymphocytes. It was demonstrated that the immunosuppressive actions of the  $\delta$ -opioid antagonists are not mediated by any of the three  $\mu$ -,  $\delta$ -, or  $\kappa$ -opioid receptors.

In mitogen stimulated peripheral blood mononuclear cells (PBMC), Th1-type cytokine interferon- $\gamma$  (IFN- $\gamma$ ) activates the enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan into kynurenine. In parallel to IDO, IFN- $\gamma$  induces GTP-cyclohydrolase I, which gives rise to enhanced neopterin formation in human macrophages. In diseases that are associated with Th1-type immune activation and inflammation, accelerated tryptophan degradation manifests in increased serum neopterin and decreased tryptophan concentrations. Significant associations were detected between blood levels of IFN- $\gamma$ , neopterin and tryptophan degradation in various diseases such as human immunodeficiency virus infection, malignancy and autoimmune

syndromes.

In this study, the immunomodulatory effects of NTI and two structurally related d-opioid receptor antagonists, HS-378 and HS-459, on mitogen-induced human PBMC was investigated. PBMC were stimulated with phytohemagglutinin A or concanavalin A, and the influence of the opioid compounds on the activation-induced degradation of tryptophan was monitored in the supernatant by HPLC. In parallel to tryptophan degradation, neopterin concentrations were determined by ELISA (BRAHMS; Hennigsdorf/Berlin, Germany). All three opioid compounds significantly inhibited in a concentration-dependent manner tryptophan degradation and neopterin formation in mitogen-induced PBMC, indicating a suppressive effect on the activated T cells and the production of IFN- $\gamma$ . These data provide additional evidence for the *in vitro* immunosuppressive activity of the tested d-opioid receptor antagonists. The influence of the compounds on the cytokine-induced tryptophan degradation might be of clinical relevance for the treatment of diseases associated with an altered immune function.

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### **Cytokine Production and Oxidative Stress in Stimulated Peripheral Blood Mononuclear Cells**

Klieber M, Fischer B, Ledochowski M, Schennach H, Fuchs D

Division of Biological Chemistry, Biocentre, and Department of Internal Medicine, Innsbruck Medical University; Institute for Blood Transfusion and Immunology, University Clinics, Innsbruck, Austria

Various immunopathologic conditions are associated with elevated neopterin concentrations which indicate an activated Th1-type immune response in the patients. In parallel to neopterin production, pro-inflammatory cytokine interferon- $\gamma$  (IFN- $\gamma$ ) induces the degradation of tryptophan via enzyme indoleamine 2,3-dioxygenase.

In this study, the production of neopterin and the degradation of tryptophan in stimulated peripheral blood mononuclear cells (PBMC) were compared with other markers of immune activation like cytokines, soluble cytokine receptors and also 8-isoprostane, a marker of oxidative stress.

For our *in vitro* study we used freshly isolated PBMC from whole blood of healthy donors. The cells were seeded at a density of  $1.5 \cdot 10^6$  cells/ml. After stimulation with mitogens phytohemagglutinin (PHA) or concanavalin A (Con A) the supernatants were collected after 48 hours. The concentrations of neopterin were measured by ELISA (BRAHMS, Hennigsdorf/Berlin, Germany). Tryptophan and kynurenine concentrations were analysed by HPLC. The measurements of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), 75kD soluble TNF receptor (sTNF-R75), soluble interleukin-2 receptor- $\alpha$  (IL-2sRa), and 8-isoprostane were carried out by ELISA (R & D; Biomedica, Vienna, Austria, and Cayman, IBL, Hamburg, Germany).

Significantly higher concentrations of neopterin were found in the supernatants of stimulated cells (Con A  $21.6 \pm 2.43$  nmol/L) and PHA ( $18.5 \pm 4.11$  nmol/L; both  $p < 0.01$ ) compared to unstimulated cells ( $8.1 \pm 2.14$  nmol/L). The degradation of tryptophan was reflected in increased concentrations of kynurenine and decreased tryptophan levels, the kynurenine to tryptophan ratio (kyn/trp) was strongly enhanced (all  $p < 0.01$ ). Also concentrations of cytokines TNF- $\alpha$  and IFN- $\gamma$ , as well as soluble cytokine receptors sTNF-R75 and IL-2sRa were increased. Neopterin concentrations correlated significantly with all the markers of immune activation (all  $p < 0.01$ ). The analyses also showed higher concentrations of 8-isoprostane in stimulated cells. However the variance of results measured was rather high and the difference was only significant for the Con A-stimulated cells but not for PHA. In general, concentrations of neopterin, kyn/trp, cytokines and cytokine receptors as well as 8-isoprostane tended to be higher upon stimulation of PBMC with Con-A than with PHA.

In conclusion, our *in vitro* investigation not only confirms the well known associations between the production of neopterin and the degradation of tryptophan in mitogen-stimulated

PBMC. Close associations exist between neopterin and the other markers of immune activation like cytokines and soluble cytokine receptors and also 8-isoprostane, a marker of oxidative stress. Thus, elevated neopterin concentrations together with increased tryptophan degradation in patients suffering from immunopathologies allow the assumption that also the production of the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ , of soluble cytokine receptors and also of reactive oxygen species is increased. Their production is induced by IFN- $\gamma$  and they may contribute to the development of oxidative stress especially in chronic immunopathologies.

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### **Cytokine Expression in Circulating Monocytes in Complicated Malaria and Malarial Anaemia**

Kneringer K, Thuma P, Gordeuk VR, Mair S, Fritsche G, Weiss G

Department of General Internal Medicine, Clinical Immunology and Infectious Diseases, Innsbruck Medical University, Innsbruck, Austria; Macha Mission Hospital, Macha, Zambia; and Howard University, Washington, DC

Severe malarial anemia (SMA) and cerebral malaria (CM) are frequent complications of *Plasmodium falciparum* malaria and the major cause of mortality from this disease in children.

As changes in immune response patterns may pre-dispose for, or being associated with these complications and because severe malarial anemia cannot be simply explained by the destruction of red blood cells, we prospectively analysed cytokine expression patterns in monocytic cells obtained from children suffering from SMA (n = 70), CM (n = 33) and uncomplicated malaria (n = 70). The study was approved by the national ethical committees. We used blood that was drawn during routine laboratory examinations of children during the admission process at Macha

Mission Hospital (day = 0) and one sample during follow up (day 35). Mononuclear cells were prepared, RNA was extracted and quantitative RT-PCR for cytokine genes of interest was performed.

Children developing SMA had significantly increased interleukin-10 (IL-10) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) concentrations at day 0 than the two other groups. Cerebral malaria was associated with a significant reduction in interferon- $\gamma$  expression and reduced levels of macrophage migration inhibitory factor. In contrast, patients with uncomplicated malaria had significantly higher interferon- $\gamma$  levels at day 0 than the two other groups.

Taken together, these results might demonstrate that a stimulated Th1-type immune response is associated with protection and a beneficial clinical course, while an overwhelming innate immune activation as estimated by increased TNF- $\alpha$  and IL-10 levels leads to SMA.

### **Serum Tryptophan Concentration in Pollinosis Patients Predicts Outcome of Pollen Extract Therapy**

Kositz C, Schroecksnadel K, Grander G, Schennach H, Kofler H, Fuchs D

Division of Biological Chemistry, Biocenter, & Department of Internal Medicine, Innsbruck Medical University, and Central Institute of Blood Transfusion and Immunology, University Clinics, Innsbruck, Austria; Allergy Ambulatory, Hall/Tyrol, Austria

The immunologic background of allergic asthma and rhinitis includes a preponderance of a Th2-type immune response, which induces IgE production. In parallel, Th1-type immune response is suppressed via Th2-type cytokines like interleukin-4, -10, -13, and others. In such a downgraded Th1-type response, production of Th1-type cytokine interferon- $\gamma$  (IFN- $\gamma$ ) is diminished. As a consequence, neopterin production and tryptophan degradation in monocyte-derived macrophages or dendritic cells might be as well reduced. Therefore these latter biochemical

parameters might allow to assess the interplay between Th2-type (= humoral) and Th1-type (= cellular) immune activation.

In serum specimens of 44 atopic patients (18 women, 26 men) before any hyposensitisation treatment, neopterin concentrations were measured by ELISA (BRAHMS, Hennigsdorf/Berlin, Germany). Tryptophan and kynurenine concentrations were measured by HPLC (rp-18, phosphate buffer, flow-rate 1 ml/min; via fluorescence and UV absorption detection), kynurenine to tryptophan ratios (kyn/trp) were calculated. Results were compared to concentrations in 38 serum specimens from healthy blood donors. Finally measurements in atopics were compared with outcome of hyposensitisation treatment, on clinical grounds 27 patients were classified as responders, and 17 patients as non-responders.

Serum tryptophan concentrations were significantly higher in atopics ( $84.3 \pm 24.4 \mu\text{mol/L}$ ) than in blood donors ( $57.9 \pm 7.46 \mu\text{mol/L}$ ;  $U = 6.45$ ,  $p < 0.001$ , Wilcoxon test), kynurenine and kyn/trp were not different between the 2 groups. Although all of the neopterin concentrations measured in patients were within the normal range of healthy controls ( $< 8.7 \text{ nmol/L}$ ), average neopterin concentrations were found to be somewhat higher in atopics ( $5.93 \pm 1.89 \text{ nmol/L}$ ) than in blood donors ( $4.59 \pm 1.02 \text{ nmol/L}$ ;  $U = 3.91$ ,  $p < 0.001$ ). When comparing subgroups according to outcome of hyposensitisation therapy, non-responders has significantly higher tryptophan concentrations ( $95.7 \pm 27.0 \mu\text{mol/L}$ ) than responders ( $77.1 \pm 19.9 \mu\text{mol/L}$ ;  $U = 2.45$ ,  $p = 0.01$ ). No further significant differences of marker concentrations were observed between groups. Notably, no significant relationship was found between neopterin concentrations and tryptophan metabolic changes.

In conclusion serum tryptophan levels were higher in atopics compared to blood donors. Higher tryptophan levels may result from subnormal degradation rate, which could probably be due to suppressed Th2-type immunity and lower IFN- $\gamma$  and IDO activity in atopics. Contrary to our expectations, neopterin was also higher in atopics vs. blood donors. However, all neopterin results measured were within the earlier defined normal range of healthy controls (=95<sup>th</sup> percentile). Thus,

the significance of this finding is still unclear. Importantly, higher tryptophan concentrations were found in non-responders to hyposensitisation therapy compared to responders. Data point to a role of more severe Th2-type activity in non-responders which might explain the lower rate of tryptophan degradation. Although there was a considerable overlap of tryptophan concentrations in these two groups of patients, the measurement of tryptophan may present an option to predict the outcome of pollen extract therapy.

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### **Measurements of Neopterin, $\beta$ -amyloid and Total Tau in Cerebrospinal Fluid of Patients with Cognitive Decline**

Leblhuber F, Wichart I, Neurauter G, Schroecksnadel K, Brunschuetz D, Ranner-Staudinger P, Fuchs D

Department of Gerontology, Landesnervenklinik Wagner Jauregg, Linz Austria; Department of Neurology, Wien-Lainz, Austria; Division of Biological Chemistry Biocentre, Innsbruck Medical University, Innsbruck, Austria

Amyloid- $\beta$  ( $A\beta$ ) oligomers impair synaptic function in addition to inflammation and oxidative stress in Alzheimer's disease (AD).  $A\beta_{42}$  is a CSF marker for plaque formation. Tau protein, located in neuronal axons, with six different isoforms, has numerous phosphorylation sites. Total tau (T-tau, ELISA) is a marker of neuronal and axonal degeneration, phosphorylated tau (ELISA) is a marker of formation of tangles. T-tau ( $237 \pm 12 \text{ pg/ml}$ ) is found 300% increased in AD versus normal controls (NC) with 84% sensitivity and 91% specificity.  $A\beta_{42}$  ( $650 \pm 12 \text{ pg/ml}$ ) is found 50% reduced in AD versus NC with 89% sensitivity and a specificity of 90%. Addition of P-tau can differentiate AD from other dementias with more than 80% specificity. There is high predictive value of prodromal AD in MCI (95% sensitivity, 83-87% specificity) for different combinations of CSF biomarkers. Plaques and tangles

(highly insoluble) as well as damaged cells in AD cortex are classical inflammatory stimuli from the earliest to the terminal stages of AD, not surprising that inflammation occurs in AD.

Apolipoprotein E (ApoE), a cholesterol transporter, is a polymorphic gene with three alleles of varying frequency (ApoE3 78%, ApoE4 15%, ApoE2 7%). ApoE4 is less efficient in reuse of membrane lipids and neuronal repair. In sporadic cases of AD, ApoE 4 allele increases dementia by 3 times in heterozygotes and by 15 times in homozygotes. Each ApoE 4 copy lowers age of onset of dementia by about 10 years. ApoE4 correlates to reduced  $A\beta_{42}$  in CSF.

In this pilot study 111 patients with cognitive deficits were investigated: AD (n = 58), mild cognitive impairment (MCI; n = 40), Lewy body dementia (LBD; n = 4), Huntington's Chorea (HC; n = 5), and vascular dementia (VD; n = 4). ApoE 4 alleles were found in 32/111 cases: ApoE 4/2 (6) ApoE 4/3 (18) ApoE 4/4 (8). No correlation was found between ApoE 4 and CSF markers  $A\beta_{42}$  and T-tau and CSF neopterin concentration. Correlation was seen between CSF neopterin and T-tau ( $p = 0.03$ ), indicating that biochemical changes of neuronal degeneration in CSF, reflected by increased T-Tau, correlated with neopterin as a marker of neuroinflammation in MCI as well as in different forms of dementia. As anti-Tau drugs are still preclinical, could anti-inflammatory treatment (NSAIDs) possibly be an option in MCI and prodromal AD in midlife?

#### **Attenuation of Microvascular Reperfusion Injury Following Murine Pancreas Transplantation by Tetrahydrobiopterin**

Maglione M, Oberhuber R, Hermann M, Schneeberger A, Mark W, Obrist P, Werner-Felmayer G, Werner ER, Margreiter R, Brandacher G

Department of General and Transplant Surgery, Center for Islet-Cell Transplantation, Institute of Pathology and Division of Biological Chemistry, Biocenter, Medical University Innsbruck, Austria

Tetrahydrobiopterin ( $BH_4$ ) is an essential

cofactor for nitric oxide synthases and thus a critical determinant of NO production. Recently we have shown that  $BH_4$  depletion contributes to ischemia reperfusion injury (IRI) after pancreas transplantation. In this study we investigated the therapeutic potential of  $BH_4$  supplementation during organ retrieval and the early post-transplant period.

Male syngeneic C57BL6 (H-2b) mice, 10-12 weeks old were used as size-matched donor and recipient pairs. Murine cervical heterotopic vascularized pancreas transplantation was performed with a modified no-touch technique. Pancreatic grafts were subjected to 16 hours prolonged cold ischemia (CIT) time and different treatment regimens: untreated (I),  $BH_4$  160 $\mu$ M to perfusion solution (II),  $BH_4$  50mg/kg i.m. at reperfusion (III). Non transplanted animals served as controls (IV). After 2h of reperfusion intravital fluorescence microscopy was used for analysis of graft microcirculation by means of functional capillary density (FCD) and capillary diameters (CD). Quantitative assessment of inflammatory responses (mononuclear infiltration) and endothelial disintegration (edema formation) was done by histology (H&E) and peroxynitrite formation was assessed by nitrotyrosine-immunostaining.

After prolonged CIT FCD was significantly reduced, paralleled by an increased peroxynitrite formation, when compared with controls (all  $p < 0.05$ ). Microcirculatory changes correlated significantly with intragraft peroxynitrite generation (Spearman:  $r = -0.56$ ;  $p < 0.01$ ). Pancreatic grafts treated with  $BH_4$  either during retrieval (II) or systemically (III) displayed markedly higher values of FCD ( $p < 0.01$ ) and abrogated nitrotyrosine staining ( $p < 0.05$ ). Histological evaluation showed increased inflammation, interstitial edema, hemorrhage, acinar vacuolization and focal areas of necrosis after 16h CIT in group I, which could be diminished by both  $BH_4$  treatment regimens ( $p < 0.05$ ).

$BH_4$  treatment, either during organ retrieval or in the early post-transplant period, significantly reduces post-ischemic deterioration of microcirculation as well as histologic damage and peroxynitrite formation and might be a promising novel strategy in attenuating IRI in clinical pancreas transplantation.

### **Food Colorants and Preservatives Inhibit Neopterin Production and Tryptophan Degradation *in vitro***

Maier E, Fischer B, Ledochowski M, Fuchs D

Division of Biological Chemistry, Biocentre, and Department of Internal Medicine, Innsbruck Medical University, Innsbruck, Austria

Food additives like preservatives or colorants are in widespread use. Their harmlessness is widely tested especially in animal model systems, and so the food additives are usually considered to be safe. However, their effect on health especially on the immune system is discussed quite controversially, and especially food colorants are very often suspected to carry some risk for allergic responses.

In this *in vitro* study, the impact of four food preservatives, sodium benzoate (E211), methylparaben (E218), propionic acid (E280) and sodium lactate (E325), and of four colorants, curcumin (E100), naturally product curcuma, tartrazine (E122) and azorubin (E102) on unstimulated and mitogen-stimulated peripheral blood mononuclear cells (PBMC) was investigated. Cell culture supernatants from  $1.5-3.0 \times 10^6$  cells/ml were collected 48 hours after they were stimulated or not with the mitogen phytohemagglutinin (Sigma, Vienna, Austria) and exposed to compounds. To monitor stimulation of T-cells, neopterin production and tryptophan degradation by indoleamine 2,3-dioxygenase (IDO) were assessed, both biochemical pathways are induced by the pro-inflammatory cytokine interferon- $\gamma$ .

When stimulated PBMC were treated with curcumin, benzoate or propionate, a significant and dose-dependent suppression of neopterin production and tryptophan degradation was observed ( $p < 0.05$ ). Also tartrazine and azorubin had a suppressive effect on stimulated PBMC, whereas sodium lactate and curcuma had only slight or no influence on these biochemical pathways. Methylparaben only suppressed tryptophan degradation in stimulated cells, whereas any effect on neopterin production did not reach statistical significance. In unstimulated cells, preservatives and colorants had more heterogenous

effects on neopterin production and tryptophan degradation: curcumin suppressed neopterin production and tryptophan degradation, whereas azorubin, tartrazine, and benzoate only suppressed the spontaneous neopterin production, whereas tryptophan degradation was even increased at certain concentrations. Methylparaben enhanced neopterin production and tryptophan degradation in parallel. Finally, curcuma, lactate and propionate had only slight or no effects on unstimulated cells.

Data indicate a suppressive effect of most food additives on the stimulated Th1-type immune response. This anti-inflammatory potential of compounds could even contribute to some health benefit of food additives, they could reduce disease activity of inflammatory conditions like viral infections, autoimmune disorders, cardiovascular and neurodegenerative diseases and even malignancy, which are associated with signs of Th1-type immune activation such as elevated neopterin levels and enhanced tryptophan degradation. However, they may also increase the risk of developing malignancy and infections, when anti-proliferative effector functions induced by interferon- $\gamma$  are hampered. Moreover, excessive intake of these anti-inflammatory substances may not only result in inhibition of Th1-type immune response but also in a shift of the Th1-Th2 immune balance towards overwhelming Th2 response. As a consequence, the probability of developing atopic diseases like allergy and asthma would increase even if food additives do not necessarily serve as allergens themselves. Further investigations on the dose-dependent immune modulation of food additives are required to validate these hypotheses.

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**Preatherosclerosis and Metabolic Abnormalities in Obese Juveniles During Lifestyle Intervention - the Impact of SAT Distribution - The STYrian Juvenile Obesity Study (STYJOBS)**

Mangge H, Almer G, Sargsyan K, Nemet A, Spindelboeck W, Dolinar U, Horejsi R, Möller R, Windhaber J, Schober P, Truschnig M

Clinical Institute for Medical and Chemical Laboratory Diagnosis, and Institute for Medical Chemistry, Medical University of Graz, Austria

Obesity is dramatically increasing in the western world, whereby juveniles are affected in particular. Atherosclerosis, a major consequence of obesity, starts early in life and results in cardiovascular disease and stroke, the main causes of mortality in industrialized countries. The STYrian Juvenile OBesity Study (STYJOBS) is a prospective study to improve the understanding of atherosclerosis and metabolic changes in obesity by investigation of the "non-biased" early phase. Based on this information an early intervention was started.

Aims of this study were the identification of "individual high risk patterns" in juvenile obesity by linking lab parameters (adipokines, 60 lipid subfractions, molecular genetics), individual adipose tissue topography (Lipometry), early vascular changes (Carotis sonography), life style habits, sport performance diagnostics, and clinical data. STYJOBS-Databank, to perform an early intervention by a holistic strategy (i.e. Lipometer adjusted Sport Program, Nutritional Device, Behavioural Therapy). STYJOBS-Intervention (40 obese juveniles, duration of intervention 12 months) and to establish a serum/plasma/DNA resource of obese kids, their parents, and normal weight age matched controls for advanced research (e.g. molecular genetics) in juvenile obesity: STYJOBS-Biobanking.

So far we investigated 350 obese juveniles (age  $13.1 \pm 4.3$  years) and 250 normal weight, age matched healthy controls. Currently, >300 variables per study participant are available. Laboratory data (e.g. orale glucose tolerance test, clotting parameters), biomarkers (ELISA, HPLC etc.), carotis sonography [carotis communis arteries, intima-media thickness (IMT), and diameter], bicycle ergometry, and adipose tissue topography were obtained

Obese juveniles exhibit an increased IMT of CCA accompanied by a low grade inflammation.

Adipokine patterns and IMT correlated with subcutaneous adipose tissue distribution as determined by lipometry. Dyslipidemia ( $\beta$ -quantification) is associated with symptoms of oxidative stress as detected by increased lipid peroxidation, incipient type II diabetes, NASH and increased systolic blood pressure. After 6 months of intervention the obese juveniles showed a significant decrease in percentual body fat, BMI-SDS, homocysteine, blood pressure, and oxidized LDL. Juveniles with trunk weighted SAT distribution showed less effective metabolic improvements concerning insulin resistance, and HDL-cholesterol increase. The most striking difference was observed in HOMA-index levels. The pear-type kids showed a highly significant shift near normal levels whereas the apple-type kids showed even an increase of HOMA index after six months of intervention. Furthermore, after intervention the clotting parameters (e.g. endogenous thrombin potential, lag-time) changed towards an increased excitability in the apple group whereas the pear kids reacted in a total different way.

Our data underline the close relationship between juvenile obesity, inflammation, incipient type II diabetes, hypertension, oxidative stress, dyslipidemia, fatty liver disease and preatherosclerosis. This pathology, and the dysregulation of adipokines is closely linked to the SAT-tissue topography. Interventional Strategies against obesity should consider individual different responses possibly linked to the SAT-distribution.

### **The Catalytic Properties and Structure of Phenylalanine Hydroxylase from the Cold Adapted Bacterium *Colwellia psychrerythraea*** 34

Martinez A, Leiros HKS, Pey A, Moe E, Inneset M, Leiros I, Steen I

University of Bergen, N-5009 Bergen, Norway; and The Norwegian Structural Biology Centre (NorStruct), University of Tromsø, N-9037 Tromsø, Norway

We here report on the catalytic properties and the structure-function relationships of phenylalanine hydroxylase from the psychrophile bacteri-

um *Colwellia psychrerythraea* 34H (CpPAH). The gene encoding the enzyme has been cloned, expressed and the resulting protein purified and kinetically characterized followed by crystal structure determination. CpPAH displayed maximal activity at 25°C (when measured at 5 mM L-Phe and 0.5 mM tetrahydrobiopterin (BH<sub>4</sub>) cofactor). *C. psychrerythraea* includes enzymes supporting BH<sub>4</sub> synthesis and recycling, but it was interesting to investigate other cofactors in their capacity to sustain CpPAH activity. The relative activities with the other cofactors, also assayed at 0.5 mM, were 20%, 17% and 7% of the activity with BH<sub>4</sub> for tetrahydrofolate, 6,7-dimethyltetrahydropterin and (6R)-5,6,7,8-tetrahydro-L-monapterin, respectively, indicating that BH<sub>4</sub> is a probable natural cofactor for CpPAH. Compared to mesophilic bacteria and mammalian organisms CpPAH showed high K<sub>m</sub>-values for the substrate ( $S_{0.5}$ (L-Phe) = 1.16 ± 0.11 mM, at 10°C) and tetrahydrobiopterin cofactor (K<sub>m</sub> = 61 ± 7 μM at 10°C) and high catalytic efficiency at 10°C. The temperature for maximum activity appears to be much lower than the inactivation- and half denaturation temperatures ( $T_m$ ) (~ 52.5°C). Thus, the enzyme appears to use a mechanism for cold adaptation in which the overall conformational stability is not largely affected. The crystal structure displays regions of local flexibility close to the highly water exposed binding sites for BH<sub>4</sub> (Gly87/Phe88/Gly89) and L-Phe (Tyr114-Pro118, in particular Gln116/Glu117). This local flexibility around the active site probably accounts for the low affinity the enzyme has for substrate and cofactor and the low temperature optimum for activity.

#### **Urinary Neopterin and Peripheral Blood Lymphocytes During Primary Chemotherapy with the Combination of Doxorubicin and Paclitaxel in Patients with Breast Carcinoma**

Melichar B, Tousekova M, Hornychova H, Vokurkova D, Solichova D, Vesely P, Jandik P, Mergancova J, Urmanska H, Ryska A.

Departments of Oncology and Radiotherapy, Medicine, Immunology, Pathology, Gerontology

& Metabolic Care, Surgery, and Radiology, Charles University Medical School & Teaching Hospital, Hradec Králové, Czech Republic

In recent years, there has been considerable interest in the study of alterations of the immune system of breast carcinoma patients undergoing primary chemotherapy. We have investigated urinary neopterin and peripheral blood leukocyte phenotype in breast carcinoma patients treated with primary chemotherapy by the combination of doxorubicin and paclitaxel before the start of the treatment and on 3 subsequent visits in approximately monthly intervals. Urinary neopterin was measured by high-performance liquid chromatography and peripheral blood phenotype was determined by flow cytometry. Compared to controls, patients with breast carcinoma had significantly lower relative number of CD3<sup>+</sup> lymphocytes, and significantly higher relative and absolute numbers of NK cells, relative and absolute numbers of CD3<sup>+</sup>DR<sup>+</sup> lymphocytes, relative and absolute numbers of CD3<sup>+</sup>CD69<sup>+</sup> lymphocytes, relative numbers of CD8<sup>+</sup>CD57<sup>+</sup> lymphocytes and CD14<sup>+</sup>DR<sup>+</sup> monocytes and relative and absolute numbers of CD14<sup>+</sup>CD16<sup>+</sup> monocytes. Significant correlations were observed before the initiation of therapy between urinary neopterin and the relative numbers of CD3<sup>+</sup>CD4<sup>+</sup> lymphocytes ( $r_s = -0.41$ ,  $p < 0.01$ ), absolute numbers of CD3<sup>+</sup>CD4<sup>+</sup> lymphocytes ( $r_s = -0.31$ ,  $p < 0.05$ ), relative number of CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes ( $r_s = 0.35$ ,  $p < 0.05$ ), CD4<sup>+</sup>/CD8<sup>+</sup> ratio ( $r_s = 0.40$ ,  $p < 0.01$ ), relative numbers of CD3<sup>+</sup>DR<sup>+</sup> lymphocytes ( $r_s = 0.34$ ,  $p < 0.05$ ), absolute numbers of CD3<sup>+</sup>DR<sup>+</sup> lymphocytes ( $r_s = 0.41$ ,  $p < 0.01$ ), and relative numbers of CD8<sup>+</sup>CD57<sup>+</sup> lymphocytes ( $r_s = 0.31$ ,  $p < 0.05$ ). Compared to pretreatment values, urinary neopterin, relative and absolute numbers of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> lymphocytes were significantly increased throughout the course of treatment. In contrast, the relative and absolute numbers of CD19<sup>+</sup> lymphocytes and CD19<sup>+</sup>CD23<sup>+</sup> lymphocytes were significantly decreased throughout the course of therapy. The relative and absolute numbers of monocytes were significantly increased throughout the course of therapy.

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### Urinary Neopterin in Patients with Ovarian Cancer

Melichar B, Urbanek L, Krcmova L, Kalabova H, Svobodova I, Dragounova E, Vesely P, Hyspler R, Solichova D

Departments of Oncology & Radiotherapy, Medicine, Gerontology & Metabolic Care, and Gynecology & Obstetrics, Charles University Medical School & Teaching Hospital, Hradec Kralove, Czech Republic

Urinary neopterin, an indicator of systemic immune activation, is increased in most patients with epithelial ovarian carcinoma (EOC) and is an independent prognostic indicator. The data on prognostic significance of neopterin in EOC have been collected before the advent of paclitaxel that has changed the management and natural history of the disease. In the present study, we have evaluated the prognostic significance of urinary neopterin in 49 patients with primary and secondary ovarian neoplasms treated in the late 1990s and in 2000s. Urinary neopterin was measured by high-performance liquid chromatography. Compared to controls, urinary neopterin was significantly increased in patients with both primary ovarian cancer and ovarian metastases of other tumors ( $341 \pm 343$ , and  $328 \pm 277$  vs.  $133 \pm 40$   $\mu\text{mol/mol}$  creatinine;  $p < 0.001$ ). Serious toxicity of chemotherapy was observed in 8 out of 12 (67%) patients with urinary neopterin equal or above  $338$   $\mu\text{mol/mol}$  creatinine (mean of all patients) compared to 2 of 19 (11%) of patients with urinary neopterin below  $338$   $\mu\text{mol/mol}$  creatinine (Fisher exact test,  $p = 0.001$ ). No significant changes were observed in urinary neopterin concentrations during the treatment with paclitaxel/platinum. A significant correlation was observed between urinary neopterin and percentage of xylose absorbed ( $r_s = -0.58$ ,  $p < 0.05$ ), and positive correlations were observed between urinary neopterin and lactulose/mannitol ( $r_s = 0.63$ ,  $p < 0.05$ ), lactulose/xylose ( $r_s = 0.79$ ,  $p < 0.001$ ) and

sucrose/xylose ( $r_s = 0.60$ ,  $p < 0.05$ ) ratios. Survival was significantly longer in patients with urinary neopterin below  $338$   $\mu\text{mol/mol}$  creatinine in the whole group of 49 patients with ovarian cancer, in 36 patients with primary ovarian cancer as well as in 13 patients in ovarian metastases of other primary tumors. A significant difference in survival was also observed when 37 pretreated patients or 24 pretreated EOC patients were evaluated ( $p = 0.05$ ). In conclusion, neopterin remains a significant prognostic indicator in patients with recurrent ovarian cancer in the era of newer chemotherapeutic agents. Increased urinary neopterin was associated with chemotherapy toxicity.

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### Changes in Plasma Neopterin in Hematology Diseases

Mistrik M, Parrak V, Sujanova Z, Skrakova M, Zwiewka M, Fuchs D

Clinic of Hematology and Transfusiology, University Hospital Bratislava, Bratislava, Slovakia; University Medical Innsbruck, Innsbruck, Austria

The answer to the question of cellular immunity activation is important for diagnosis and optimal management of many hematology diseases. Hematology abnormalities due to immune-mediated attack occur as an isolated condition involving mature cell only (e.g. red blood cell, neutrophils, platelets), as a progenitor-specific cytopenia manifested by absence of some or all progenitor forms in bone marrow (lineage specificity), or in association with other cytopenias. The aim of study is to look at the cellular immunity status in these disorders via neopterin concentration estimation.

In our study, 95 patients (50 males, 45 females), median age 51 years (20 - 81), with diagnosis autoimmune thrombocytopenia (AITP = 31), aplastic anemia (AA = 12), autoimmune

haemolytic anaemia (AIHA = 5), Status post allogeneic stem cell transplant (alloSCT = 7), chronic lymphocytic leukemia (CLL = 36), and control group (non immune anaemia = 4) were included. The number of serum samples analysed was 144. Neopterin was estimated with Enzyme-Linked Immunosorbent Assay (ELISA, BRAHMS, Berlin, Germany) (reference value:  $5.3 \pm 2.7$  nmol/L). Statistics performed by SPSS (Kolmogor-Smirnov test, T-test, Wilcoxon test).

Significant neopterin elevation ( $p < 0.05$ ) was observed in patients after allogeneic SCT, in patients with AA and CLL progression or infectious complication. Serum neopterin level correlated with AITP and AIHA response to immunosuppressive therapy. There was tendency toward elevated neopterin level in active AIHA phase ( $p = 0.056$ ).

Immunosuppressive therapy is double-edged sword, very effective in management of immune-mediated hematology disorders, but opening area for infectious complications at the same time. Getting more information about disease activity before apparent cytopenia could have a major impact on patient disease free survival and quality of life.

### **Mechanism of Action Studies with the Antitumor Agent Calcium-pterin**

Moheno P, Winkler C, Pfeleiderer W, Barral AM, Scuderi, R, Carlson J, Fuchs D

SanRx Pharmaceuticals, Inc., La Jolla, California, USA; Faculty of Chemistry, University of Konstanz, Germany; La Jolla Institute for Allergy and Immunology, San Diego, California, USA; The Scripps Research Institute, La Jolla, California, USA; University of California San Diego, California, USA; Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck, Austria

Previous studies by us have shown a strong antitumor effect for orally-administered (1:4 mol:mol) and (1:2 mol:mol) calcium-pterin (TGI = 70%), and for dipterinyl calcium pentahydrate (DCP) (TGI = 78%); as well as unexpectedly for

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (TGI = 74%) in nude mice with MDA-MB-231 human breast tumor xenographs. No toxicity, as determined by body weight loss  $> 10\%$ , was observed with DCP. Several lines of evidence indicate that calcium-pterin acts as an immunomodulator. A stepwise regression analysis of nine serum cytokine and indoleamine 2,3-dioxygenase (IDO) metabolite changes in the nude mice identified four which were correlated to calcium-pterin administration: 1) decreased IL-6, 2) increased IL-10, 3) decreased IFN- $\gamma$ , and 4) increased kynurenine.

*In vitro* studies in human PBMCs found that calcium-pterin decreased the activity of IDO and the production of neopterin. In this system, pterin-complexed  $\text{Ca}^{+2}$  showed a substantially greater inhibition of IDO activity and neopterin production than either  $\text{Ca}^{+2}$  alone or pterin alone. In cytotoxicity studies, calcium-pterin caused an initial activation of NK cells followed by NK cell inhibition with Day 3 effectors. FACS analysis of PMA-stimulated splenocytes indicated that calcium-pterin increased the expression of IFN- $\gamma$  in macrophages at 4 hrs, which subsided at 24 hrs.

Calcium-pterin exerts a significant dose-response antitumor activity in nude mice with MDA-MB-231 xenographs ( $r_s = -0.83$ ;  $p = 0.013$ ), and causes immunological changes which implicate a Th1 to Th2 shift involving dendritic cells, T regulatory cells, and/or macrophages, as well as the suppression of the NF- $\kappa\text{B}$  system of subcellular signal transduction. DCP, with its greater antitumor efficacy, is hypothesized to have a similar mechanism of action to calcium-pterin. Planned binding studies are expected to identify and characterize specific DCP receptor(s) among these cell types.

### **Asymmetric Dimethylarginine (ADMA) and Neopterin in Patients with and without Angiographic Coronary Artery Disease**

Murr C, Meinitzer A, Seelhorst U, Wellnitz B, Hallwachs-Baumann G, Schroecksnadel K, Mangge H, Boehm BO, Winkelmann BR, März W, Fuchs D

Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck,

Austria; Clinical Institute of Medical and Chemical Laboratory Diagnostics, Graz Medical University, Austria; Synlab Center of Laboratory Diagnostics, Heidelberg, Germany; Division of Endocrinology, Department of Medicine, University Hospital, Ulm, Germany; Cardiology Group Sachsenhausen, Frankfurt am Main, Germany; LURIC Database LLC, Freiburg, Germany

Asymmetric dimethylarginine (ADMA), a competitive inhibitor of the nitric oxide synthases, is produced by methylation of arginine residues in intracellular proteins by the enzyme arginine N-methyltransferase. Elevation of circulating ADMA concentration is associated with an increased vascular tone and blood pressure and is considered to play a pivotal role in endothelial dysfunction. Neopterin is produced by human monocyte-derived macrophages upon stimulation with Th1-type cytokine interferon- $\gamma$  and is a sensitive indicator for cellular immune activation and also oxidative stress due to immune activation.

In a cross-sectional approach, blood concentrations of ADMA, homocysteine, neopterin, folic acid and vitamins B6 and B12 were compared in 2030 patients, which were recruited within the Ludwigshafen Risk and Cardiovascular Health (LURIC) study.

ADMA concentrations were not significantly different in patients with coronary artery disease (mean  $\pm$  SD:  $0.82 \pm 0.15$   $\mu\text{mol/L}$ ) and controls ( $0.81 \pm 0.14$   $\mu\text{mol/L}$ ; Welch's t test:  $p = \text{n.s.}$ ). In contrast, patients with coronary artery disease had higher homocysteine (mean  $\pm$  SD:  $14.1 \pm 6.2$   $\mu\text{mol/L}$ ) and neopterin ( $8.5 \pm 7.3$   $\text{nmol/L}$ ) concentrations compared to controls (homocysteine:  $12.6 \pm 5.1$   $\mu\text{mol/L}$ , neopterin:  $7.5 \pm 4.8$   $\text{nmol/L}$ ; both  $p < 0.0001$ ). There was a significant positive correlation between ADMA concentrations and homocysteine ( $r_s = 0.207$ ), neopterin ( $r_s = 0.276$ ; both  $p < 0.0001$ ) and vitamin B12 ( $r_s = 0.061$ ;  $p < 0.01$ ) and a significant inverse correlation with vitamin B6 ( $r_s = -0.190$ ;  $p < 0.0001$ ) and folic acid ( $r_s = -0.063$ ;  $p < 0.01$ ) concentrations.

Data do not show an association of coronary artery disease with increased ADMA concentrations, but higher ADMA production is associated with an increase of homocysteine and neopterin

production in patients at risk for atherosclerosis. The correlation found between ADMA and neopterin concentrations is in line with recent *in vitro* findings which showed that also stimulation of peripheral blood mononuclear cells is associated not only with production of neopterin but also of ADMA. Data imply that ADMA production may be influenced by homocysteine production and oxidative stress due to immune activation.

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#### Neopterin Production, Tryptophan Degradation and Cancer

Neurauter G, Fischer B, Ledochowski M, Fuchs D

Division of Biological Chemistry, Biocenter, and Department of Internal Medicine, Innsbruck Medical University; and Central Institute of Blood Transfusion and Immunology, University Hospital Innsbruck, Innsbruck, Austria

Enzyme indoleamine 2,3-dioxygenase (IDO) is inducible by interferons and converts the essential amino acid tryptophan to N-formyl-kynurenine. Induction of IDO is considered as one out of several anti-proliferative pathways which are

induced during immune response and which are directed to halt reproduction of pathogens in infected cells and to stop malignant growth. In 1998, researchers around Andrew Mellors and David Munn demonstrated that enhanced IDO activity is critical in the induction of immunotolerance during pregnancy (1), and interest in IDO was rapidly spread into several labs throughout the world. Meanwhile, numerous studies of basic regulation and consequences of IDO activation broadly extended the knowledge and understanding of its potential clinical relevance. Results demonstrated a central place of IDO in the development of immunodeficiency and immunotolerance. IDO activation has also been claimed as an intrinsic tumoral immune resistance mechanism. However, degradation of tryptophan in unstimulated tumor cells is observed only rarely *in vitro*. Clinical studies are able to support an important role of accelerated tryptophan degradation in human diseases, which has been described, e.g., in cytokine-treated patients and in a variety of disorders such as infections including HIV infection, in autoimmune syndromes, in malignant diseases, in cardiovascular disorders and in neurodegeneration (2). These diseases often go along with inflammation and immune activation, and significant associations were observed between the kynurenine to tryptophan ratio (kyn/trp; an estimate of tryptophan degradation) and markers of Th1-type immune activation such as neopterin or soluble cytokine receptors. Accordingly, accelerated tryptophan degradation was referred to enhanced IDO activity which is due to endogenous formation of its primary inducer, namely interferon- $\gamma$ . In the above-mentioned clinical conditions, striking associations exist between accelerated tryptophan degradation and the extent, the activity and the course of the diseases: in malignancy and in HIV infection, accelerated catabolism of tryptophan and immune activation predict shorter survival and concur with the loss of immunocompetence and the development of cachexia and anemia. Data suggest a pathogenetic role of tryptophan deprivation also in these symptoms, which are typical for chronic inflammatory conditions. Also in cancer patients, the accelerated tryptophan degradation is unlikely a spontaneous activity of tumor cells, rather it is

induced by surrounding immunocompetent cells as part of their anti-tumoral immune defense strategy, which in the long run becomes detrimental when tryptophan deprivation is strong enough to diminish T-cell responsiveness (3). Thus, enhanced tryptophan degradation is part of the cytotoxic activity of the activated immune system, however it is also an important aspect in the development of immunodeficiency when the immune system fails to eradicate the malignant process in, e.g., cancer patients. The same two-edged situation is true in autoimmunity: accelerated tryptophan degradation is demonstrated along with the disease course, however, the immunosuppressive capacity of activated IDO is unable to counteract the destructive autoimmune process.

All together, *in vivo* data support a role of IDO in the complex interplay between immune activation cascades and the development of immunotolerance, apoptosis and immunodeficiency, as well as anemia and neuropsychiatric symptoms. They result from the pro- and anti-inflammatory consequences of Th1-type cytokine interferon- $\gamma$ , among which IDO is a central player.

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#### HPLC Determination of Serum Phenylalanine and Tyrosine via Monitoring of their Natural Fluorescence

Neurauter G, Ledochowski M, Mayersbach P, Schennach H, Fuchs D

Division of Biological Chemistry, Biocenter, Department of Internal Medicine, Innsbruck Medical University, Central Institute of Blood Transfusion and Immunology, Innsbruck University Clinics, Innsbruck, Austria

Essential aromatic amino acid phenylalanine (Phe) is precursor of tyrosine (Tyr) and thus important for the biosynthesis of several neurotransmitters like dopa, dopamine, noradrenaline and adrenaline. Hydroxylation of Phe into Tyr is achieved by enzyme phenylalanine-4-hydroxylase (PAH), of which 5,6,7,8-tetrahydrobiopterin (H<sub>4</sub>Bip) is an important cofactor as hydrogen donator. Patients with PAH- or H<sub>4</sub>Bip-deficiency present with phenylketonuria (PKU) which is detected after birth by screening for elevated Phe concentrations in the blood. Significantly increased serum Phe concentrations, however at a far lesser extent than in PKU patients, are observed also in other clinical conditions, e.g. in patients with HIV, cancer or trauma (1). These conditions are known to go along very often also with elevated neopterin concentrations, and earlier we have reported on a correlation between neopterin and Phe in patients post-trauma (Roth et al, 2003; this meeting series). To be able to address the question if serum Phe concentrations are increased in several clinical conditions which are associated with immune activation and with elevated neopterin concentrations, we created an HPLC method to quantify serum concentrations of Phe and Tyr simultaneously and with high sensitivity.

Because of their natural fluorescence, which is much more sensitive than UV detection, both amino acids can be sensitively measured at an excitation wavelength of 210 nm and an emission wavelength of 302 nm. For each HPLC analysis an albumin-based calibration mixture containing 5 µmol/L Tyr and 20 µmol/L Phe is prepared. 100 µl of standard, pool serum (external standard) or sample, 100 µl 50 µM L-nitrotyrosin and 2M trichloroacetic acid are vortexed and centrifuged with 12000 g for 6 minutes at room temperature to precipitate the proteins. Supernatants of samples and external standards are diluted 1:25 as Tyr has an upper detection limit of 6 µmol/L and serum Tyr concentrations are about 100 µmol/L.

With a flow rate of 0.9 ml/min at room temperature and an injection volume of 55 µl one run is completed within 8 minutes.

Acknowledgement: Supported by the Stiftung Propter Homines, Vaduz-Fürstentum Liechtenstein.

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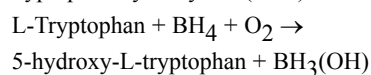
### **Immunohistochemical and Enzymological Detection of Tryptophan hydroxylase in Villus Epithelium of Intestinal Mucosa**

Ohashi A, Nakamura K, Sato T, Hasegawa H

Department of Biosciences, Teikyo University of Science and Technology Department of Pathology, Juntendo University School of Medicine

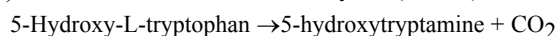
Serotonin (5-hydroxytryptamine, 5HT) is synthesized from L-tryptophan through two steps of enzyme reaction:

(I) Tryptophan hydroxylase (TPH):



by

(II) Aromatic-L-aminoacid decarboxylase (AADC):



TPH is the rate limiting enzyme in 5HT biosynthesis and localized in 5HT-synthesizing tissues. TPH exists in two isoforms (TPH1, TPH2) and they express differently in the body. Strong expression of TPH1 is known in peripheral organs such as the pineal gland and GI (gastrointestinal) tract. On the other hand, TPH2 is dominantly expressed in CNS. We first demonstrated TPH quantitatively in the GI mucosa and distinguished it from the neural TPH by immunoprecipitation assay using an antiserum raised against TPH purified from mouse mastocytoma cells P-815 (1987). Later, the antiserum revealed considerable con-

densation of TPH in the enterochromaffin cells of GI tract and in the mast cells.

Recently, we prepared antibodies mono-specific to TPH1 and TPH2, both raised against regional oligopeptides close to N-terminal of least homology between TPH1 and TPH2. The antibodies revealed uneven localization of TPH1- and/or TPH2- immunoreactive signals (IRS) in various cells of most organs. Uneven condensation of TPH1- and TPH2-IRS drew our attention at apical region of villus epithelium of intestinal mucosa. Hence we challenged to break through the great gap in the sensitivity between immunohistochemistry and enzymology: we tried to demonstrate TPH activity quantitatively in the villus region. However, cell-free TPH assay to detect the enzyme product 5HTP by FD-HPLC was failed with isolated brush borders or Caco-2 cells, a representative cell line of intestinal epithelium derived from human colon adenocarcinoma. We finally quantitatively detected *de novo* synthesis of 5HT in Caco-2 cells under monolayer culture. The production was stimulated by BH<sub>4</sub> enriched as a form of sepiapterin, the precursor of BH<sub>4</sub>. The role of 5HT or its precursor 5HTP synthesized locally at the micovilli inside the epithelia is under investigation.

### **High-sensitive Procalcitonin (PCT) in the Diagnosis of Neonatal Infection**

Parrak V, Secnik P, Greksova K, Venczelova Z, Mistrik M, Stencl P, Scholz E, Fuchs D

St. Cyril and Method Univeristy hospital Bratislava, Slovakia, Division of Biological Chemistry, Division of Medical Biochemistry, Biocenter, and Department of Clinical Nutrition, Innsbruck Medical University, Innsbruck, Austria

Newborn infants often suffer from bacterial and viral infections without presenting typical symptoms. Therefore, reliable methods for detecting and monitoring bacterial or viral infection, or/and sepsis in the newborn would be beneficial. In many studies, procalcitonin (PCT) and other serum markers as neopterin and high sensitivity C-reactive protein (hsCRP) have proved to be use-

ful to support diagnosis of infection and inflammation. We have evaluated the concentrations of PCT using chemiluminescence assay PCT and PCT-Sensitiv (both from BRAHMS, Henningsdorf/Berlin, Germany) and ELISA for neopterin (BRAHMS) in mothers before delivery and in cord blood at delivery. Samples were frozen by minus 25°C. The inclusion criteria for healthy mothers and neonates were term delivery, gravidity without antibiotic therapy, without clinical symptoms and signs of infection, and mother and neonate to leave the hospital within 4 days without any complications. Gestational age was 40 ± 2 weeks, weight 3500 ± 500 g, height 50 ± 2 cm, Apgar score 9 ± 1. In January 2007, 106 samples were collected from mothers and their cord blood, 84 fulfilled entry criteria, and data were compared them to a similar group of patients which were collected in October and November 2001. Using PCT-Sensitiv, average PCT concentrations were 0.033 pg/ml in 2001 mothers and 0.016 in 2007 mothers, 0.081 in 2001 cord blood and 0.086 in 2007 cord blood. PCT concentrations with "regular" PCT assay were 0.103 in 2001 mothers and 0.157 in 2001 cord blood. Results suggest that the measurement of PCT-Sensitiv need to be evaluated and compared with old data of the "normal" PCT assay. Average neopterin concentrations were 7.36 nmol/L in 2001 specimens measured in 2007 as compared to 7.01 nmol/L measured in 2002. Average neopterin concentrations were 6.23 in 2007 specimens. In 2001 cord blood neopterin concentrations were 16.99 nmol/L measured in 2007 and 16.08 measured in 2002. In 2007 cord blood specimens, neopterin concentrations were 16.48 nmol/L.

Reference ranges still need to be established in all compartments including mother, cord blood and the neonate. It is expected that simultaneous evaluation of these markers may be of use in differentiating between bacterial and viral etiologies.

### **Modulatory Effects of Asian Multi-compound Medicine on Stimulated Peripheral Blood Mononuclear Cells *in vitro***

Pittl S, Fischer B, Jenny M, Ueberall F, Fuchs D

Division of Biological Chemistry, Division of

Medical Biochemistry, Biocenter, and Department of Clinical Nutrition, Innsbruck Medical University, Innsbruck, Austria

In ancient times herbs were used in rituals for health maintenance and for the treatment or prevention of diseases many of which are of chronic or inflammatory nature. Asian multi-compound herbal formulas are believed not only to help to cure the immediate disease but also take care not to have negative side effects. So, there are many positive prospects and challenges in doing research on multi-compound Asian medicine ahead. It is definitely important to elucidate the effectiveness and the active principle of multi-compounded formulas. In this way the benefits of eastern medicine can be made accessible for people living outside Asia, and also Asian community can learn and benefit from the research, as it helps to increase the acceptance of traditional Asian Medicine in the West. It seems reasonable that multi-compounded herbal formulas play a role to counteract inflammation and modulate the immune system. Within cellular (= Th1-type) immune response, which is initiated during a variety of diseases like virus infections, autoimmune syndromes, and malignant, cardiovascular or neurodegenerative disorders, enzymes indoleamine 2,3-dioxygenase and GTP-cyclohydrolase I are specifically induced by cytokine interferon- $\gamma$ . Therefore cellular immune activation can be monitored by determination of neopterin concentrations and tryptophan degradation. This can be applied also *in vitro* by using peripheral blood mononuclear cells (PBMC). Earlier we have described an anti-inflammatory effect of Tibetan herbal multi-component Padma 28 (1).

In this *in vitro* study, the modulatory effect of multi-compounded Asian medicine on mitogen-stimulated PBMC was investigated. PBMC from whole blood of healthy donors were seeded at a density of  $1.5 \cdot 10^6$  cells/ml. Cells were pre-incubated with the compounds, which were dissolved in culture medium. Mitogen phytohaemagglutinin A (PHA, 10 $\mu$ g/ml) was added and cells were incubated for 48 hours at 37°C and 5% CO<sub>2</sub>. Increased neopterin production and tryptophan degradation was found in stimulated PBMC, and both biochemical pathways were significantly

suppressed by some of the multi-compounded plants, e.g., from Triphala (*Terminalia chebula*, *Terminalia bellerica*, Amla), and Ling Zhi. The Amla extract had more effect on the tryptophan degradation than on neopterin formation.

The tested plant extracts are able to counteract inflammatory response in a dose-dependent way but significant effects were achieved only at relatively high concentrations. Since both pathways, tryptophan degradation and neopterin production were influenced by the extracts simultaneously, Th1-type immune response is likely to be suppressed by reducing the expression of the cytokine interferon- $\gamma$ . Antioxidant compounds seem to be important for this activity (2). They may be responsible for or at least contribute to the healthy benefits which are ascribed to these multi-component traditional medicines.

Acknowledgement: Supported by the "Stiftung Propter Homines, Vaduz-Fürstentum Liechtenstein.

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#### **Influence of Cacao Extracts on Neopterin Production and Tryptophan Degradation in the Myelomonocytic Cell Line THP-1**

Santer E, Wondrak A, Jenny M, Ledochowski M, Ueberall F, Fuchs D

Division of Biological Chemistry, Division of Medical Biochemistry, Biocenter, and Department of Clinical Nutrition, Innsbruck Medical University, Innsbruck, Austria

Chocolate, especially the dark kind, is considered to have a variety of potential health benefits particularly in context of vascular function. These

effects appear to be related almost exclusively to the content of cacao in chocolate. Earlier, cacao extracts have been found to suppress neopterin production and tryptophan degradation via indoleamine 2,3-dioxygenase (IDO) in stimulated peripheral blood mononuclear cells (PBMC) (Jenny M, et al., this meeting). Since both these effects are mediated by the Th1-type cytokine interferon- $\gamma$ , the suppressive action of cacao extracts most likely relates to a down-regulation of activated T-cells. However, a direct effect of cacao extracts on macrophages cannot be excluded, e.g., earlier we have described that HMGCoA-reductase inhibitor atorvastatin not only suppressed activated T-cells but also had a direct influence to suppress neopterin production and tryptophan degradation in the myelomonocytic cell line THP-1 (1).

In this study, it was investigated whether water or ethanol (30%) cacao extracts are also able to reduce neopterin production and tryptophan degradation in myelomonocytic THP-1 cells.

Cells were seeded at a density of  $1 \times 10^6$  cells/ml and were pre-incubated with both aqueous and ethanolic cacao extracts. Cells were then stimulated with  $1 \mu\text{g/ml}$  lipopolysaccharide (LPS) and after 48h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  the supernatants were collected. Neopterin concentrations were measured by ELISA (BRAHMS). Tryptophan and kynurenine concentrations were determined by high performance liquid chromatography (HPLC) via fluorescence und UV absorption detection.

Analysis showed an increased production of neopterin and enhanced degradation of tryptophan in LPS-stimulated THP-1 cells compared to unstimulated cells. However, none of the two extracts had any influence on the stimulated monocyte cells, neither neopterin production nor tryptophan degradation were suppressed or stimulated further.

In conclusion, unlike atorvastatin the suppressive influence of cacao extracts on stimulated PBMC seems due to an exclusive effect on the cytokine production by the stimulated T-cell population, which induces formation and release of neopterin and tryptophan degradation in the monocyte-derived macrophages.

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### **Muscle Trauma and Immune Activation after Exhaustive Endurance Exercise**

Schobersberger W, Sumann G, Greie S, Griesmacher A, Falkensammer G, Hoffmann G, Fuchs D, Koller A

Institute for Leisure, Travel and Alpine Medicine, University for Health Sciences, Medical Informatic and Technology, Hall/Tyrol, Division for General and Surgical Intensive Care Medicine, and Division of Biological Chemistry, Biocentre, Innsbruck Medical University; Central Institute for Medical and Chemical Laboratory Diagnostics, and Institute for Sports Medicine, University Hospital, Austria

Prolonged exercise can be associated with multiple changes in the immune status mainly indicating to an activation of the immune system. At present it is unknown if the type and duration of exercise as well as the training status influences the degree of immune activation. Moreover we speculated that eccentric muscle activation (e.g. downhill running) induces micro-traumata of skeletal muscles thus inducing a pronounced inflammatory response. Data in the literature focusing on post-exercise changes in s-neopterin were heterogenous. Either no changes were described (e.g., Berlin Marathon, triathlon, 1 h submaximal bicycle test) or small increases were reported (Oetztal bicycle marathon, Swiss Alpine Marathon, alpine hiking tour). At present there are no data to which extent the immune system is activated after a downhill marathon run. Thus, we analysed (A) the literature regarding neopterin and exercise and (B) immune parameters and parameters of muscle damage before and after a 42 km downhill marathon run.

Thirteen volunteers participated at the Tyrolean Speed Marathon (42 km downhill race, 700 m vertical displacement; mean running time 3.43

hrs). Blood from antecubital veins was collected 3 days (T1) and 3 hrs (T2) before the run, within 30 min after finishing (T3) and one day thereafter (T4). Measured parameters were neopterin, white blood picture, creatine kinase (CK), myoglobin. All parameters were corrected for plasma volume changes. In addition before and one day after the race an isokinetic muscle test was performed. Statistics: Friedman ANOVA, Wilcoxon test and Spearman's rank correlation coefficients. Data are given as mean values  $\pm$  SD.

Results revealed during the Tyrolean Speed Marathon were as follows: Total CK and myoglobin were elevated at T3 and were further increased at T4 (mean CK and myoglobin values at T4: 4879 U/L and 1409  $\mu$ g/ml). Baseline serum neopterin was  $5.7 \pm 0.9$  nmol/L. At T3 neopterin was significantly increased by 2.1 nmol/L. It was still increased at T4. There was a reduction in peak hamstring torque (both thighs) indicating to eccentric hamstring fatigue. We did not find any correlations between neopterin and markers of micro-muscle damage or neopterin and parameters of isokinetic dynamometry.

Increases of neopterin after prolonged exercise are an inconstant finding. In case of a significant increase this elevation was only moderate as compared to infectious diseases. There is obviously no correlation between exercise-induced changes in neopterin and type and duration of exercise or training status. We could demonstrate that a 42 km downhill marathon induces an activation of the cellular immune system, as evidenced by a significant elevation of neopterin. However, the absolute changes in neopterin were moderate and similar to other types of exercise (bicycle marathon, mountain hiking). These changes were accompanied by pronounced increases in markers for skeletal muscle damage; however there was no correlation between neopterin and the degree of skeletal muscle trauma.

#### **Amino acid CSF/Plasma-ratios in Children**

Scholl-Bürgi S, Haberlandt E, Heinz-Erian P, Albrecht U, Baumgartner-Sigl S, Rauchenzauner M, Karall D

Department for Pediatrics, Medical University Innsbruck, Innsbruck, Austria

Cerebrospinal fluid (CSF)/plasma-ratios for amino acids may elucidate transport mechanisms for amino acids at the blood-brain-barrier *in vivo*. However, to date no reference values for amino acid CSF/plasma-ratios in childhood are available. The aim of the study was therefore to establish these reference values. Beside others, amino acid concentrations for essential neutral amino acids (threonine, valine, methionine, leucine, isoleucine, phenylalanine and tyrosine) in CSF and plasma were measured in 68 patients with ion-exchange chromatography and ninhydrine detection (70 measurements, age 3 days - 17.2 years) for routine metabolic work up. Multiple linear regression analysis was performed to evaluate the influence of antiepileptic drug therapy, age and sex on CSF/plasma-ratios.

The CSF/plasma-ratios for valine, isoleucine, leucine and tyrosine showed a significant age dependency, valine, methionine and phenylalanine were influenced by valproate-therapy and phenylalanine was further influenced by phenobarbital-therapy. Sex showed no influence on CSF/plasma-ratios. The established reference values were in the same range as results from literature (for adults) and different from those of patients with known genetic disorders and inborn errors of metabolism.

The amino acid CSF/plasma-ratios should be determined in routine metabolic work up in patients with neurologic symptoms of unknown origin. They may help elucidate the pathomechanisms in known disorders or detect new disorders, like inborn errors of amino acid transport at the blood-brain-barrier.

#### ***In vivo* and *In vitro* Immune Response in Patients with Bacterial Infection**

Schroeksadel K, Fuchs D, Keller M, Grubeck-Loebenstein B, Bellmann-Weiler R, Hammerer-Lercher A, Weiss G

Department of General Internal Medicine, Clinical Immunology and Infectious Diseases;

Department for Laboratory Medicine, and Division of Biological Chemistry, Biocentre, Innsbruck Medical University; Academy for Aging Research, Innsbruck, Austria

Earlier it was shown that peripheral blood mononuclear cells isolated from patients with human immunodeficiency virus (HIV) infection have a decreased ability to respond to stimulation *in vitro*. The switch to a predominant cellular immune response during the course of HIV infection might be involved in this phenomenon: Interferon- $\gamma$  (IFN- $\gamma$ ), which is the key cytokine of Th1-type immune response, induces monocytes to form neopterin and in parallel degrade tryptophan via indoleamine (2,3)-dioxygenase (IDO). IDO activation is well established to efficiently suppress T-cell proliferation. Additionally, plasma neopterin concentrations as marker for the *in vivo* immune activation status were recently shown to be suited well to estimate the capacity of HIV infected PBMC to respond to stimulation *in vitro*.

This study examined IFN- $\gamma$  -mediated pathways and immune activation status of 24 patients with bacterial infection *in vivo* (by determination of CRP, neopterin, blood counts) and compared them to the proliferative activity of peripheral blood mononuclear cells (PBMC) *in vitro*. 16 patients were followed up 1-3 weeks after their admittance as out-patients. PBMC of younger and older patients suffering from bacterial infection were stimulated with mitogens concanavalin A, phytohemagglutinin and pokeweed mitogen. Neopterin concentrations and tryptophan degradation were measured in plasma as well as supernatants of unstimulated and stimulated cells, proliferation rate was assessed by <sup>3</sup>H-thymidine incorporation.

As expected, inflammation and immune activation markers were elevated in patients with bacterial infection and declined when patients recovered. Patients showed a higher degree of IDO-activation than controls. Stimulation with mitogens induced cell proliferation and in parallel, enhanced neopterin formation and tryptophan degradation *in vitro*. During bacterial infection, PBMC of patients showed a significantly stronger proliferative response to Con A stimulation, than PBMC isolated from healthy controls. In healthy

controls and convalescent patients, respectively, proliferative response to Con A and PHA-stimulation was best in individuals younger than 30 years, while older patients showed a declining capacity to proliferate. Age was furthermore also associated with inflammation markers, iron metabolism and enhanced tryptophan degradation. Furthermore, also CRP and neopterin concentrations were related with the proliferative response to mitogens. According to these data, age and also inflammation markers are a major determinant of proliferative response *in vitro*.

### **Increased Asymmetric Dimethylarginine (ADMA) Formation by Mitogen-stimulated Peripheral Blood Mononuclear Cells**

Schroeksadel K, Weiss G, Stanger O, Teerlink T, Fuchs D

Department of General Internal Medicine, and Division of Biological Chemistry, Biocentre, Medical University of Innsbruck, Austria; Department of Cardiac Surgery, Atherosclerosis Research, Paracelsus Medical University, Salzburg, Austria; Metabolic Laboratory, Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands

Elevated concentrations of asymmetric dimethylarginine (ADMA) as well as of total homocysteine in the blood are established to reflect an increased cardiovascular risk. ADMA is an endogenous inhibitor of NO-synthase, which is produced by endothelial cells. Elevated ADMA concentrations often coincide with hyperhomocysteinemia.

Earlier human peripheral blood mononuclear cells (PBMC) were found to produce homocysteine upon stimulation with mitogens concanavalin A, phytohaemagglutinin and pokeweed mitogen. In this study, the ability of PBMC to form ADMA and symmetric dimethylarginine (SDMA) was determined. Effects were compared to levels of cysteine, homocysteine and arginine in cultures. Increased concentrations of ADMA and SDMA were found in mitogen-stimulated compared with unstimulated PBMC. Arginine

and cysteine concentrations did not differ between stimulated and unstimulated PBMC. There existed significant associations between concentrations of homocysteine and ADMA ( $r_s = 0.575$ ) as well as SDMA ( $r_s = 0.436$ , both  $p < 0.001$ ). Treatment of stimulated PBMC with the anti-inflammatory compounds salicylic acid (5 mM) and atorvastatin (25  $\mu$ M) decreased the rate of ADMA and SDMA formation. Results of these *in vitro* studies show that formation of asymmetric dimethyl-arginines coincides with homocysteine production in human PBMC. Thus, activated PBMC not only release Th1-type cytokine interferon- $\gamma$ , which is the most important inducer of nitric oxide synthase, but also ADMA, a natural inhibitor of the enzyme. As stimulation of PBMC also induces neopterin production, our *in vitro* findings may explain the close association which is usually found between ADMA and neopterin concentrations in, e.g., patients with coronary disease. It would be interesting to investigate, whether patients suffering from other chronic diseases, which present with elevated neopterin concentrations, also show increased ADMA levels.

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#### Iron Perturbation Affect Cellular Immunity via Modulation of TIM Expression

Schroll A, Moschen A, Nairz M, Theurl M, Weiss G

Department of General Internal Medicine, Clinical Immunology and Infectious Diseases, Medical University of Innsbruck, Innsbruck, Austria

The T cell immunoglobulin and mucin proteins (TIM) - are a novel gene family, which are expressed on immune effector cells. Their different expression during differentiation and proliferation of T helper (Th) precursor cells into T effector lineages is of major importance for generating

the Th1 and Th2 subtypes, respectively. Tim-1 is expressed on recently activated T cells and provides a costimulatory signal for Th2 cell differentiation and proliferation. Tim-2 can be found on Th2 cells and has two different ligands, namely ferritin and Sema4A. The interaction of Tim-2 and its ligands plays an important role in immune regulation by negatively affecting the Th1 response. Tim-3 can be detected on terminally differentiated Th1 cells, and the interaction with its ligand galectin9 leads to their apoptosis via an increased calcium flux. The relationship between Fe status, immune function, and cellular resistance to infection has been the subject of a number of studies. On the one hand, macrophages play a key role in iron homeostasis during infection by increasing iron retention. On the other hand, iron has distinct effects on cytokine production and on the antimicrobial defence strategies of macrophages, which alter lymphocytes as well as macrophages activation and proliferation.

To investigate the effects of iron perturbation on Tim-1, Tim-2 and Tim-3 mRNA expression *in vivo*, 10 C57bl/6 mice were divided into 4 groups. 5 mice received an iron diet, whereas the others were kept on normal diet. After 2 weeks, 3 mice of the iron group and 3 mice of the non-iron group were challenged with heat-inactivated *Salmonella typhimurium* by intraperitoneal injection. All mice were sacrificed 3 days post infection and cytoplasmatic mRNA levels of Tim1, Tim-2, Tim-3, IL-4 and interferon- $\gamma$  (IFN- $\gamma$ ) from CD4 positive sorted spleen cells were determined by quantitative real-time PCR. Additionally, mRNA levels of iNOS, TNF- $\alpha$  and IL-10 from liver tissue were determined by RT-PCR. Iron-load mice did not show a qualified TNF- $\alpha$  and iNOS response to *S. typhimurium* challenge. Moreover, iron loaded mice exhibited a Th2 response, which was shown by IL-4 up-regulation, Tim-3 down-regulation and Tim-2 up-regulation during challenge.

*In vitro*, CD4 sorted spleen cells from C57bl/6 mice were stimulated with antiCD3/antiCD28 + PMA/ionomycin and challenged with an iron salt (FeSO<sub>4</sub>, 50 $\mu$ M) or the iron chelator desferrioxamine (DFO, 50 $\mu$ M) for 48 hours. Cytoplasmatic mRNA levels for Tim-1, Tim-2 and Tim-3 were determined by quantitative real-time PCR. Iron chelation by DFO leads to a down-regulation of IFN- $\gamma$  and Tim-3, but to an increased mRNA

expression of Tim-1. Thus, iron interferes in the Th1/Th2 balance by inducing a Th-2 response pattern which may be referred to direct regulation of Tim expression by the metal both *in vitro* and *in vivo*.

### Intake of Alcoholic Beverages on Urinary Neopterin Excretion

Schubert C, Fuchs D

Medical Psychology and Psychotherapy, and Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck, Austria

Antioxidant vitamins like vitamin C and E or plant and fruit compounds like resveratrol have been found to suppress neopterin production and tryptophan degradation in mitogen-stimulated peripheral blood mononuclear cells *in vitro* (1). In a similar way, extracts of plants like green and black tea and also alcoholic beverages like red and white wine and beer were found to do the same (2, 3).

Applying an integrative single-case design we determined neopterin concentrations in five women (three suffering from SLE, two healthy), who collected their entire urine on a twice-daily basis throughout 8 weeks. Aside from the central interest of our studies, i.e., the impact of daily stressors on cellular immune system dynamics, subjects also provided information on their intake of alcoholic beverages (type and quantity) during the study period. We tested for a possible statistical association between the daily intake of alcoholic beverage and the fluctuations of urinary neopterin concentrations using time series analysis (i.e., ARIMA modelling, cross-correlational analysis). The mean urine neopterin levels were 199 (Case 1 SLE), 204 (Case 2 SLE), 229 (Case 3 SLE), 124 (Case 4 healthy), and 194 (Case 6 healthy)  $\mu\text{mol/mol}$  creatinine. In two of the five cases the intake of alcoholic beverages was significantly correlated with urine neopterin levels: In SLE Case 1, neopterin decreased 12 hours after the intake of alcoholic beverages (lag0:  $r = -0.407$ ;  $p < 0.05$ ); in SLE Case 3, neopterin decreased within 12 hours after drinking (lag0:  $r = -0.197$ ;  $p < 0.05$ ); and in Case 4 (healthy),

neopterin decreased 24 hours after the intake of alcoholic beverages (lag2:  $r = -0.205$ ;  $p < 0.05$ ). In Case 2 (lag0:  $r = -0.143$ ; n.s.) there was a tendency that neopterin decreased within the first 12 hours after drinking. These data suggest that intake of alcoholic beverages may be followed by a decline of neopterin concentrations within approx. 12 to 24 hours. Notably, urines collected after 12 hours or 24 hours reflect the influence of beverages in the preceding 12 hours. Interestingly, the highest level of significance was observed in Case 1 with SLE who had reported the highest frequency and amount of alcohol intake (approx. 0.3 L sparkling wine/d) in the study period. Results agree well with the *in vitro* findings that alcoholic beverages are able to suppress neopterin formation in stimulated peripheral blood mononuclear cells (2, 3). *In vitro* data also support the conclusion that antioxidant compounds like resveratrol in wine or humulones in beer rather than ethanol are responsible for the immunomodulatory influence (1). Data support the view that such beverages do possess some potential to improve inflammatory conditions or to reduce cardiovascular risk. Naturally, only moderate consumption can be recommended to avoid adverse effects that ethanol at larger dosage may have. Alternatively, the use of non-alcoholic beer is an option without alcohol-related risks. At least *in vitro* rather similar effects on the stimulated blood cells were seen using regular and non-alcoholic beer (1-4). Further studies are needed to confirm the *in vivo* relationship between intake of alcoholic beverages and decline of urinary neopterin concentrations.

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### Testing Cellular Immune Activity in a Breast Cancer Survivor under Real-life Conditions

Schubert C, Geser, W, Fritzsche K, Burbaum C, Neises M, Fuchs D, Schmid-Ott G

Medical Psychology and Psychotherapy, and Division of Biological Chemistry, Biocentre, Innsbruck Medical University, and Institute of Psychology, University of Innsbruck, Innsbruck, Austria; Department of Psychosomatic Medicine, University of Freiburg, Freiburg, Germany; Department of Psychosomatic Medicine, Hannover Medical School, Hannover, Germany

This integrative single-case study investigated the influence of emotionally meaningful daily incidents on cellular immune system dynamics in a breast cancer survivor.

The 60 year-old patient with breast cancer (primary diagnosis 5 years before study start, cancer recurrence 1 year before study start, disease free at study start) collected her entire urine for a period of 32 days in 12-hour intervals. In addition, she filled out questionnaires regarding her emotional state, daily routine (e.g., medication) and illness perception. Weekly interviews (IHI) identified the past week's incidents. After the end of the study, neopterin (cellular immune parameter, HPLC) was measured in the 63 consecutive urine samples. Time series analysis consisted of ARIMA modeling and cross-correlational analysis.

Mean urine neopterin level was 164  $\mu\text{mol/mol}$  creatinine. Daily stressors were followed by a cyclic response pattern in neopterin, i.e. a decrease after 24 hours (+lag2: -0.237;  $p < 0.05$ ) and an increase after a total of 84 hours (+lag7: +0.272;  $p < 0.05$ ). On the other hand, medication (i.e., anastrozole, zinc orotate, selenite, Cinnabaris, sodium sulfur) was first associated with an increase in neopterin after 12 hours (+lag1: +0.237;  $p < 0.05$ ) and then with a decrease after a total of 60 hours (+lag5: -0.242;  $p < 0.05$ ). The patient did not suffer from sickness behavior.

When recent findings on patients with SLE and

healthy probands were considered, the results on this patient suggest a dysfunctional stress system with increased cellular immune activity/inflammation when stressed. The fact that the patient showed no signs of sickness behavior and normal urine neopterin levels may have been related to suppression of inflammatory activity by daily medication.

### Tetrahydrobiopterin and FDG microPET in Hypoxic Ischemic Encephalopathy in Neonatal Pigs

Shintaku H, Fujioka H, Nozaki S, Wada Y, Watanabe Y, Kajita T, Yamano T

Department of Pediatrics, and Department of Physiology, Osaka City University Graduate School of Medicine, Osaka Japan; Molecular Probe Dynamics Laboratory, Molecular Imaging Research Program, RIKEN Frontier Research System, RIKEN, Kobe Japan

Hypoxic-ischemic encephalopathy (HIE) is induced by intrauterine or perinatal distress and causes neuronal cell loss that presents clinically as either motor dysfunction, stupor, seizure or other neurological sequelae. It is generally accepted that free radicals, including nitric oxide (NO), play an important role in the pathophysiology of ischemic neuronal death. Recently, it has been reported that decreased levels of tetrahydrobiopterin ( $\text{BH}_4$ ) may also cause neuronal cell loss in HIE. In this study, we investigated the change in plasma and brain  $\text{BH}_4$  levels using a newborn piglet hypoxic ischemic (HI) model and evaluated FDG microPET imaging in the brain.

The animals used in this study were piglets a few days old with a mean weight of 2 kg. The animals were ventilated with a mixture of 6% oxygen and 94% nitrogen, followed by clamping of the bilateral common carotid arteries for 45 minutes. The clamps were then removed and the animals resuscitated with pure oxygen. These HI animals represented the experimental model while sham-treated animals served as controls. Blood samples were obtained at 0, 4, 8 and 12 hours after resuscitation. Plasma biopterin and neopterin levels were determined using high per-

formance liquid chromatography after iodine oxidation in acidic conditions.

In plasma, biopterin concentrations after HI insult increased more at 0 h ( $377.9 \pm 78.7$  nM) than before HI insults ( $80.1 \pm 4.3$  nM). The values of plasma biopterin peaked at 4 h ( $604.8 \pm 200.9$  nM) and slightly decreased at 12 h ( $445.9 \pm 57.8$  nM). Plasma neopterin was not detectable in any sample from experimental piglets. However, the biopterin concentration in the cerebral cortex did not increase until 12 hours after HI insults. The brains of the experimental animals on gross examination appeared edematous but no cysts were observed at 12 h. In microPET imaging, HI insult significantly reduced the intense (18)F-FDG uptake by brain tissue after 24 h.

We identified the imbalance of biopterin levels between plasma and brain tissue in the acute phase of HI insult in the cerebral cortex. MicroPET imaging showed low activity in brain tissue after HI. It seemed to enlarge neuronal damage because of the increase in superoxide production due to insufficiency of BH<sub>4</sub> production in the brain.

#### **A Study on Putative Oxidative Stress Markers in Patients with Thyroid Tumor**

Sipahi H, Girgin G, Sahin TT, Yuksel O, Taneri F, Baydar T

Department of Toxicology, Faculty of Pharmacy, Hacettepe University; Department of Surgery, Faculty of Medicine, Gazi University; Ankara, Turkey

The thyroid, a doubled-lobed structure located in the neck, is defined as one of the largest endocrine gland in the body. The gland produces thyroxine and triiodothyronine that regulate the rate of metabolism in the humans and are known to aid cellular proliferation. Thyroid tumors can be benign or malignant. Over production of oxygen species cause a decrease in the efficiency of antioxidant defense mechanism such as superoxide dismutase (SOD), and catalase (CAT) and play a role in the pathogenesis of various diseases. It is known that neopterin production provides prognostic information in patients with

malignant diseases. Neopterin measurement can provide information about the extent of cellular immune activation and oxidative stress. In our study, neopterin and biopterin levels and SOD and CAT activities were investigated in patients with benign and malignant thyroid disorders. Urinary neopterin and biopterin levels and also CAT and SOD activities were measured in patients with (n=20) malignant and (n=44) benign thyroid tumor. The mean levels of urinary neopterin and biopterin in patients with thyroid malignancy were  $168.5 \pm 92.04$  and  $82.62 \pm 63.81$  mmol /mol creatinine, respectively. In patient with benign tumors, urinary neopterin and biopterin levels were  $128.4 \pm 45.93$  and  $64.17 \pm 40.07$  mmol /mol creatinine, respectively. In patients with malignancy, the CAT activity was  $0.7 \pm 0.08$  U/mg protein while SOD activity was  $4.35 \pm 2.29$  U/mg protein. Patients with benign thyroid tumors, CAT activity was  $0.72 \pm 0.05$  U/mg protein and SOD activity was  $6.66 \pm 0.05$  U/mg protein. The slight elevation observed in the levels of neopterin and biopterin levels in malignant patients was not statistical significant (both,  $p > 0.05$ ). On the other hand, there was no statistically significant difference in CAT activities among the groups ( $p > 0.05$ ). The slight increase observed in SOD activity was not significant ( $p > 0.05$ ).

#### **Serum Phenylalanine, Tyrosine, Neopterin and Isoprostane in Patients with Ovarian Carcinoma**

Sperner-Unterweger B, Grahmann AV, Neurauter G, Klieber M, Zeimet A, Ledochowski M, Fuchs D

Division of Biological Chemistry, Biocentre, and Departments of Psychiatry, Gynaecology, and Internal Medicine, Innsbruck Medical University, Innsbruck, Austria

The aromatic amino acid phenylalanine is essential for humans and is substrate for phenylalanine (4)-hydroxylase (PAH). PAH produces tyrosine, another important amino acid which is precursor for the biosynthesis of DOPA and catecholamines dopamine, epinephrine and norepi-

nephrite. Earlier increased blood levels of phenylalanine were reported in patients with HIV infection, with cancer and in trauma and sepsis. All these clinical conditions are known to be linked with inflammation and immune activation and with increased concentrations of immune activation marker neopterin. Oxidative stress due to chronic immune activation and inflammation could be involved in the increase of serum phenylalanine concentrations in patients. It could be due to a reduced conversion rate of phenylalanine to tyrosine by PAH, which should be reflected by an increased phenylalanine to tyrosine ratio (phe/tyr), an estimate of PAH activity.

In 61 patients with ovarian cancer, serum concentrations of phenylalanine and tyrosine were measured by HPLC. In parallel, markers of inflammation and immune activation were determined by ELISA. Phenylalanine concentrations were found to correlate with concentrations of interleukin-6 ( $r_s=0.277$ ), interleukin-2 receptor- $\alpha$  ( $r_s=0.270$ ), soluble 75kD TNF-receptor ( $r_s=0.250$ ) and neopterin ( $r_s=0.319$ ; all  $p < 0.05$ ). Phe/tyr correlated even stronger with these markers: interleukin-6 ( $r_s=0.394$ ), interleukin-2 receptor- $\alpha$  ( $r_s=0.305$ ), neopterin ( $r_s=0.374$ ; all  $p < 0.01$ ) and soluble 75kD TNF-receptor ( $r_s=0.254$ ,  $p < 0.05$ ). In addition, phenylalanine concentrations ( $r_s=0.231$ ,  $p < 0.05$ ) and phe/tyr ( $r_s=0.376$ ,  $p < 0.01$ ) correlated with isoprostane concentrations.

Correlations between phenylalanine and phe/tyr and several immune activation markers point to a potential role of inflammation and immune activation in the accumulation of phenylalanine. Moreover, correlation existed between phe/tyr and isoprostane, a marker of oxidative stress. Thus, reactive oxygen species formed during chronic immune activation and inflammation may diminish tetrahydrobiopterin, the oxidation-sensitive cofactor of phenylalanine hydroxylase. In turn, phe/tyr may increase as a consequence of oxidative stress due to immune activation.

In conclusion, it is well established that inflammation and immune activation may disturb biosynthesis of neurotransmitter serotonin via cytokine-induced tryptophan-degradation. Data of this study suggest that also the adrenergic nervous system might be affected, when chronic and overwhelming production of reactive oxygen species by stimulated immunocompetent cells

reduces the life-span of essential cofactor tetrahydrobiopterin. Thus, in addition to cytokine-induced tryptophan and serotonin abnormalities, also adrenergic metabolism might be disturbed by immune activation.

### **Volatile Organic Compounds Released from Lung Cancer Cells *in vitro*.**

#### **Release of Volatile Organic Compounds from Cancer Cells *in vitro*: Possible use as Tumor Markers**

Sponring A, Zebrowski W, Filipiak W, Schmid A, Wiesenhofer H, Goebel T, Fuchs D, Amann A, Troppmair J

Daniel Swarovski Research Laboratory, Innsbruck; Clinical Department for Anesthesiology, University Hospital Innsbruck, Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria

Volatile organic compounds (VOCs) are increasingly recognized for their potential use as tumor markers. Improved detection methods with increased sensitivity (GC-MS, PTR-MS, and SIFT-MS) and non-invasive sampling procedures should facilitate their use in screening efforts for the detection of cancer. A huge number of possible VOCs has been described including alkenes, ketones, aldehydes, alcohols, amines, sulfides, ethers etc., but little is known about the underlying cellular processes leading to their generation and the potential usefulness as tumor markers. Tumors are complex systems displaying a high degree of heterogeneity and contribution of non-tumor components, including cells of the immune system and possibly also microorganisms. They all may individually contribute to VOCs present in the exhaled air of a tumor patient. The aim of our planned work is to test for the existence of cancer-derived VOCs, which could serve as tumor markers in the future. Besides their identification we also want to gain insights into the underlying causes of aberrant VOC production and possibly establish a link to tumor-specific genetic alterations. Included in our analyses is a panel of well characterized lung tumor cell lines, genetically modified mouse fibroblast cells as

well as their normal counterparts.

Work performed so far with the lung cancer cell line A549 focused on establishing the experimental conditions, which allow for the unambiguous detection of tumor-derived components. Optimized cultivation is now routinely performed using microcarrier beads to achieve high cell densities in a relatively small culture volume. Aeration of cell culture vessels is strictly controlled using a standardized air mixture and defined flow rates. Our experiments also identified serum used in the culture of these cells as a prominent source of VOCs and reduction in serum concentrations or serum-free culture may be necessary in future experiments. This work also detected large amounts of VOCs originating from microcarrier beads as contributor to high background levels.

So far PTR-MS on-line measurements detected no increased VOCs from this cell line, but the use of GC-MS with sampling by SPME suggested the existence of tumor cell specific products. Future work aims at the identification of other tumor-related VOCs, which will be evaluated in the comparative analyses with additional human tumor cell lines as well as patient-derived samples.

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### **A Structural View on Tetrahydrobiopterin Binding**

Teigen K, Erlend Hodneland E; Martínez A

Department of Biomedicine, University of Bergen, Bergen, Norway

The three aromatic amino acid hydroxylases (phenylalanine, tyrosine and tryptophan hydroxylase) and nitric oxide synthase (NOS) with its three isoforms all utilize (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) as cofactor. The pterin binding site in the three hydroxylases is well con-

served and different from the binding site in NOS. All the bound BH<sub>4</sub> conformations are different than that of BH<sub>4</sub> free in solution at neutral pH (based on results from NMR). The different bound conformations appear to result from specific interactions with non conserved amino acids at the BH<sub>4</sub> binding sites of the hydroxylases, notably the stretch 248-251 (numeration in PAH) and the residue corresponding to Ala322 in PAH, i.e. Ser in TH and Ala in TPH. Based on analysis of molecular interaction fields, we discuss the selectivity determinants for each hydroxylase and explain the high affinity inhibitory effect of 7-tetrahydrobiopterin specifically for PAH. We also present a method for estimating the accessible volume surrounding BH<sub>4</sub> when bound to the different proteins. A relatively large accessible volume in a particular receptor might indicate that this receptor has a unique cavity that might be exploited to develop a selective BH<sub>4</sub> analogue. Our results yield new insights into the specificity of cofactor binding to these protein families.

### **Molecular Mechanism for Pterin-mediated Inactivation and Aggregation of Tyrosine Hydroxylase**

Urano F, Hayashi N, Arisaka F, Murata S, Ichinose H

Institute of Liberal Arts and Sciences, Nagoya University, Nagoya; Comprehensive Medical Science, Fujita Health University, Toyoake; Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama; Graduate School of Environmental Studies, Nagoya University, Nagoya

Tyrosine hydroxylase (TH) catalyzes the rate-limiting step in the biosynthesis of catecholamines and the reaction requires tetrahydrobiopterin (BH<sub>4</sub>) as a cofactor. We found that pre-incubation of recombinant human TH protein with BH<sub>4</sub> results in the irreversible inactivation of the enzyme in spite of its cofactor role.

The pre-incubation of TH with BH<sub>4</sub> at a concentration far less than the K<sub>m</sub> value toward BH<sub>4</sub> caused approximately 80% loss of the TH activity, but oxidized biopterin and dihydrobiopterin

had little effect on the TH activity. And the intensity of the fluorescence and circular dichroism spectra of TH was gradually reduced by the sequential addition of BH<sub>4</sub> without changing overall profiles. When BH<sub>4</sub> was added in large excess (850 μmol/L), the TH fluorescence was completely quenched. In addition, studies using gel-permeation chromatography and turbidity measurements demonstrated that the TH protein added BH<sub>4</sub> formed aggregate with large molecular weight. We also clarified that TH molecules associated with each other prior to the formation of large aggregate by sedimentation velocity analysis. The results suggested that BH<sub>4</sub> not only acts as a cofactor, but also accelerates the inactivation and aggregation of TH.

Furthermore, TH was protected from this pterin-mediated inactivation by dopamine. So we think that the balance of the amounts of BH<sub>4</sub> and dopamine is important for regulation of TH protein. Now, we propose a novel mechanism for regulating the amount of TH protein.

### **Utilization of Liquid Chromatography for the Monitoring of Cancer Patients**

Urbanek L, Solichova D, Melichar B, Krcmova L, Solich P

Department of Analytical Chemistry, Charles University, Faculty of Pharmacy, Hradec Králové, Czech Republic; Departments of Gerontology & Metabolic Care, Medicine and Oncology & Radiotherapy, Charles University Medical School and Teaching Hospital, Hradec Kralove, Czech Republic

High performance liquid chromatography (HPLC) is currently the most important separation method and the most important analytical technique. It allows separation, identification and quantification of different analytes in relatively large concentration range in one step. High sensitivity (depending on the type of detector) and possibility of automatization are responsible for its wide utilization in clinical practice.

In order to monitor cancer patients, concentrations of different biologically active compounds

are monitored in blood and urine samples by the means of HPLC. Retinol and α-tocopherol are endogenous antioxidants involved in the pathogenesis of different disorders, including cancer. The evaluation of blood levels of these micronutrients contributes to the clarification of their potential role in cancer prevention. Furthermore, after administration of high doses of vitamin A, serum concentrations of retinol and retinyl esters allow the assessment of intestinal mucosal damage that represents a frequent side effect of anti-cancer drugs. We have developed a method for the determination of retinol, retinol esters and α-tocopherol in human serum by the means of high performance liquid chromatography using particulate and monolithic C18 columns.

Neopterin, a marker of immune system activation, represents another compound useful in monitoring cancer patients. On-line analysis of neopterin requires a sensitive and efficient analytical system for its determination in urine. Using novel technologies in HPLC, such as new types of stationary phases, modern instrumentation or column switching technique introduces alternative approaches for the chromatographic analysis of this compound.

### **Prognostic Significance of TPA versus SCC-Ag, CEA and Neopterin in Carcinoma of the Uterine Cervix**

Volgger B, Aspisirengil C, Genser-Krimbacher E, Ciresa-Koenig A, Daxenbichler G, Fuchs D, Marth C, Windbichler G.

Department of Obstetrics and Gynecology, University Hospital Innsbruck, and Division of Biological Chemistry, Biocentre, Medical University of Innsbruck, Innsbruck, Austria

In cancer of the uterine cervix, stage, tumor size, histologic type, histology grade, presence of lymphovascular space invasion and metastases in regional lymph nodes at the time of surgery have been described as prognostically important factors. The described parameters, however, are not sufficient to accurately predict patient prognosis. Further markers would therefore be very useful in determining the patient's risk for recurrence or

death.

Tumor cells release a variety of molecules which are tumor-associated antigens (TAAs). Prognostic significance of squamous cell carcinoma antigen (SCC), tissue polypeptide antigen (TPA), carcinoembryonic antigen (CEA) and of immune activation marker neopterin in patients with cervical cancer was demonstrated earlier in independent studies. We compared the prognostic value of pretreatment concentrations of serum TPA, SCC-Ag, CEA and the urine neopterin/creatinine ratio in 138 women with cervical cancer. Median age was 52 years. Of the tumors 85% were squamous cell carcinomas, 15% adeno- or adenosquamous carcinomas. In 50 (36%) cases the tumor was FIGO stage I, 33 (24%) stage II, 44 (32%) stage III and 11 (8%) stage IV. Elevated levels of TPA were measured in 22% of patients, SCC in 68%, CEA in 42% and neopterin in 36%. All groups of patients with elevation of any of these tumor markers showed significantly worse overall survival in univariate analysis (all  $p < 0.001$ ). However, only TPA remained as independent prognostic factor in multivariate analysis additional to stage, age and histologic type.

Our findings confirm and extend results of previous studies which identified SCC-Ag, TPA, CEA and neopterin to be significantly associated with survival in univariate analysis. In multivariate analysis however, SCC-Ag, CEA and neopterin had insufficient power to predict prognosis in our collective, unlike some authors, who found also SCC-Ag, CEA and neopterin as independent predictors of survival in patients with carcinoma of the uterine cervix. Different cut-offs used and differences in the follow-up time of patients may relate to this discrepancy compared to earlier studies. Obviously in our study the correlation between the concentrations of markers SCC-Ag, TPA, CEA and neopterin and the histologic type, stage and age was too close. In our group of patients, TPA was the strongest prognostic factor and only TPA remained as a significant prognostic factor in multivariate analysis for overall survival with an increased risk of 2.2 for patients with elevated as compared to normal levels as shown by the Cox proportional hazard analysis. Pretreatment TPA serum levels may serve to identify patients at higher or lower risk as classical prognostic factors like FIGO stage, grading,

tumor volume, histologic type or age suggest.

### **Glycerol Ether Monooxygenase Update**

Werner ER, Hermetter A, Prast H, Golderer G, Werner-Felmayer G

Division of Biological Chemistry, Biocentre, Innsbruck Medical University, Innsbruck Austria  
Institute of Biochemistry, Graz University of Technology, Graz, Austria; Division of Pharmacology and Toxicology, Institute of Pharmacy, Leopold - Franzens University of Innsbruck, Innsbruck Austria

Glycerol ether monooxygenase (E.C. 1.14.16.5) cleaves glycerol ethers to glycerol and the corresponding aldehyde by the aid of 5,6,7,8-tetrahydrobiopterin. The sequence of this enzyme as well as its physiological significance are unknown. Previous reports found that the activity of this enzyme is mainly found in mammalian liver and intestine.

We have set up a novel assay of the enzyme using a novel, fluorescent glycerol ether lipid carrying a pyrene fluorophore in the side chain, i.e. 1-O-pyrenedecyl-sn glycerol. Upon incubation with enzyme activity containing homogenates, this glycerol ether lipid is cleaved to glycerol and pyrene decanoic acid. This product can be separated from the substrate by HPLC and sensitively detected with fluorescence detection. Compared to the previously most widely used kinetic assays monitoring UV absorbance, our assay is five orders of magnitude more sensitive, thus allowing the detection of activities as low as 4 fmol/min.

With this assay, we investigated the tissue distribution of the enzyme in male and female rats. We confirmed earlier reports that the highest activities are found in the liver, and that male rats have an about 3 fold higher activity in the liver as compared to female rats. We found varying amounts of tetrahydrobiopterin-dependent activity in almost all organs of the rat. Surprisingly, the sex difference is confined to the liver and not found in other organs. Activity was not uniformly distributed in the brain, but was significantly higher in cerebellum as compared to striatum or cortex. Spiking of the homogenates with rat liver

microsomes yielded a mean recovery rate of 75%, irrespective of the value of activity detected. This demonstrated that the varying degrees of activity are not caused by varying concentrations of endogenous inhibitors, but do reflect different enzyme activity levels in the various homogenates.

Our results demonstrate that tetrahydrobiopterin-dependent glyceryl ether monooxygenase is widespread throughout the rat body. This finding indicates that effects on glyceryl ether monooxygenase should be considered when investigating effects of tetrahydrobiopterin treatment or deficiency in the mammalian system.

#### **Antioxidant Capacity of Cacao Extracts and their Influence on Heme Oxygenase-1 Gene Expression in the Myelomonocytic Cell Line THP-1**

Wondrak A, Santer E, Jenny M, Fuchs D, Ueberall F

Division of Biological Chemistry, Division of Medical Biochemistry, Biocenter, and Department of Clinical Nutrition, Innsbruck Medical University, Innsbruck, Austria

Cocoa-based products exhibit many biologic actions in model systems relevant to human health. These effects appear to be related almost exclusively to the content of cocoa flavonols present in cacao powder. Experiments with mitogen-stimulated peripheral blood mononuclear cells revealed that aqueous and ethanolic (30%) extracts of commercially available cacao suppress neopterin production and tryptophan degradation (PBMC; see Jenny M, et al. this meeting). Since both these effects are mediated by the Th1-type cytokine interferon- $\gamma$ , the suppressive effect of cacao extracts most likely relates to a down-regulation of activated T-cells. Likewise, experiments with myelomonocytic THP-1 cells showed that none of the cacao extracts had any influence on lipopolysaccharide (LPS) induced neopterin production or tryptophan degradation (see Santer E, et al. this meeting). Consequently, the suppressive effect of the cacao extracts on mitogen stimulated PBMC seems to represent an exclusive effect on

cytokine production by the stimulated T-cell population, which induces formation and release of neopterin and degradation of tryptophan in monocyte-derived macrophages via Th-1-type cytokine interferon- $\gamma$ .

In this *in vitro* study, we tested the antioxidant capacity of aqueous and ethanolic (30%) extracts of cacao against reactive oxygen species in the ORAC (Oxygen Radical Absorbance Capacity)-assay and investigated the influence of cacao extracted in ethanol (30%) on heme oxygenase-1 (HO-1) expression in myelomonocytic cell line THP-1 via Western blot technique. The ORAC assay measures the oxidative degradation of fluorescein (0.0816 $\mu$ M) after being mixed with the peroxy radical generator 2,2'-azobis(2-amidino-propane)dihydrochloride (AAPH). The reaction alone is compared to the reaction in the presence of a standard antioxidant (Trolox, a vitamin E analogue) and the antioxidant sample being tested. The fluorescent intensity of the fluorescein decreases as it gets oxidized, and measurements of this intensity are taken every minute for 35 minutes after the addition of the oxidant. The oxidative decay of fluorescein is less rapid in the presence of antioxidants. A graph of the decay curve (fluorescein intensity vs. time) is generated and the area under the curve is calculated. Different concentrations of Trolox are used to make a standard curve, and test samples are compared to this. Final results for test samples are expressed as micromoles Trolox equivalents/g (TE/g). The aqueous and ethanolic extracts of cacao (0.003 to 0.025mg/ml) were demonstrated to have potent antioxidant capacity against reactive oxygen species with relative ORAC values of  $737 \pm 64$  TE/g and  $694 \pm 55$  TE/g, respectively.

For the expression analysis of HO-1, THP-1 cells were seeded at a density of  $1 \times 10^6$  cells/ml and pre-incubated with ethanolic cacao extract (0.5-10 $\mu$ g/ml) for 1h. After treatment with 1 $\mu$ g/ml LPS for 48h the cells were collected and a lysate was prepared for SDS-polyacrylamide gel electrophoresis. After blocking, the membranes were incubated with a monoclonal HO-1 or a polyclonal GAPDH primary antibody for 1h and after washing the membranes, secondary horseradish peroxidase-conjugated anti-mouse for HO-1 and anti-goat for GAPDH IgG was added and incubated for another hour. Immunoreactive protein

bands were developed using ECL-PLUS (Amersham, Piscataway, NJ) and detected by lightening a Hyperfilm. The resulting blots showed an induction of HO-1 expression with increasing concentrations of the ethanolic cacao extract. At the highest doses tested, HO-1 expression was comparable to LPS-induced HO-1 expression in THP-1 cells. However, co-treatment of cells with LPS and cacao led to an inhibition of HO-1 expression at the higher doses tested. In conclusion, the cacao-extracts demonstrated to exhibit potent antioxidant capacity but the ethanolic cacao extract exhibited a stimulating effect on the expression of HO-1 whereas LPS-induced HO-1 expression seemed to be reversed at high concentrations of cacao in THP-1 cells. Thus, HO-1 expression appears to be induced by pro-inflammatory stimuli like LPS as part of an oxidative stress response but also by the antioxidant stress which is elicited by cacao extracts.

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### **CSF Neopterin as a Diagnostic Marker for AIDS Dementia**

Hagberg L, Gisslén M, Brew B, Cinque P, Price RW, Fuchs D

Department of Infectious Diseases, Sahlgrenska University Hospital, Göteborg, Sweden; Department of Neurology, University of New South Wales, Sydney, Australia; Clinic of Infectious Diseases, San Raffaele Hospital, Milan, Italy; Departments of Neurology University of California San Francisco, USA; Division of Biological Chemistry, Biocentre,

Innsbruck Medical University, Innsbruck, Austria

The diagnosis AIDS dementia is based on a clinical symptoms and the exclusion of other diseases such as opportunistic infections in the central nervous system. Although the incidence has decreased after the introduction of effective anti-retroviral therapy, it is still a clinical problem. There are no specific laboratory markers for AIDS dementia complex. High levels of cerebrospinal fluid (CSF) HIV-RNA are often present in patients with AIDS dementia, but are sometimes also found in asymptomatic HIV infection. CSF neopterin levels above 20 nmol/L (4-5 times the normal level) have previously been suggested to predict the development of AIDS dementia.

We therefore analysed how many patients in various stages of HIV-infection reached above this level. All patients included were antiretroviral-treatment naive. We found that 28/30 (93%) of patients with AIDS dementia stage 2-4; 22/22 (100%) AIDS dementia stage 1; 15/27 (56%) AIDS dementia stage 0.5; 26/48 (54%) asymptomatic HIV with CD4 cells <50/mm<sup>3</sup>, 30/55 (55%) asymptomatic HIV with CD4 cells 50-199/mm<sup>3</sup>; 23/56 (41 %) Asymptomatic HIV with CD4 200-350/mm<sup>3</sup>; and 24/97 (25%) Asymptomatic HIV with CD4 >350/mm<sup>3</sup>; had CSF neopterin levels above 20 nmol/L. In primary HIV infection 13/19 (68 %) had CSF neopterin above this level, while in HIV-negative controls 0/50 (0%) had CSF neopterin above 20 nmol/L.

We conclude that CSF neopterin concentrations is commonly elevated above 20 nmol/L in HIV infection but could not be used for confirming the diagnosis AIDS dementia. A battery of tests including not only inflammatory markers but also markers for central nervous cell injury may improve the diagnostic specificity for AIDS dementia.