SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Regulation of retinoids and n-3 PUFAs in atopy

by Johanna Mihály

Supervisor: Dr. Ralph Rühl

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF
MOLECULAR CELL AND IMMUNE BIOLOGY
DEBRECEN, 2011

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By **Johanna Mihály**, M.Sc.

Supervisor: Dr. Ralph Rühl, Ph.D.

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Head of the Examination Committee:	Prof. Dr. Pál Gergely, member of the H. A. Sc.
Members of the Examination Committee:	Dr. Márta Széll, D.Sc.
	Prof. Dr. Ferenc Erdődi, D.Sc.

The examination takes place at the Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, the 31st of May 2011

Head of the Defense Committee:	Prof. Dr. Pál Gergely, member of the H. A. Sc
Reviewers:	Prof. Dr. Éva Remenyik, D.Sc.
	Dr. Richard Porter, Ph.D.
Members of the Defense Committee:	Dr. Márta Széll, D.Sc.
	Prof. Dr. Ferenc Erdődi, D.Sc.

The PhD defence takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen, the 31st of May 2011

INTRODUCTION

Atopy and atopic dermatitis

The term “atopy” was first coined by Coca and Cooke in 1923, the word taken from Greek meaning placelessness, out of place, unusual, special. Atopy describes the predisposition to the development of allergic diseases including bronchial asthma, allergic rhinitis and atopic dermatitis (AD) and afflicts up to 20% of the population of the industrialized countries. Atopy can be characterized by: a.) increased levels of total serum IgE (where parasitic infections or other causes have to be excluded), b.) allergen specific serum IgE c.) immediate type skin reactions. However, none of these atopic phenomena are pathognomonic for atopy and not all need to be present at the same time.

AD is a highly pruritic, chronic and relapsing inflammatory skin disease and it can commonly occur concomitantly with asthma, food allergy, allergic rhinitis or conjunctivitis based on common patomechanism. It predominantly occurs in infancy and childhood, but it can also begin later in life and persist into adulthood having a high familial occurrence. In the prevalence of AD a great worldwide variation can be observed, ranging from 0.6 % to 20.5 % and its prevalence is higher in developed countries, although the reasons are not known. Two types of AD can be distinguished: extrinsic and intrinsic types. Extrinsic or allergic AD is associated with high total serum IgE levels and the presence of specific IgE for environmental and food allergens and its incidence is 70-80 % of the patients. Intrinsic or non-allergic AD exhibits normal total IgE values and the absence of specific IgE and approximately 20 % - 30 % of the patients are involved in it, with female predominance.

Atopic dermatitis is a hereditary disease presenting a spectrum of abnormalities including permeability barrier disruption, inflammation and abnormal keratinocyte differentiation. The major function of skin is to form a barrier between the external hostile environment and the internal milieu of the host. The skin must avoid the percutaneous penetration of chemical irritants and allergens; protect the host from mechanical trauma, ultraviolet light and pathogenic microorganisms, moreover it has to prevent excessive trans-epidermal water loss hindering thus the body from desiccation. The epidermal barrier is located in the deeper part of the stratum corneum, which forms a continuous sheet of protein-enriched cells (corneocytes) connected by modified desmosomes, called corneodesmosomes and embedded in an intercellular matrix enriched in non-polar lipids and organized as lamellar lipid layers. The lipid lamellae matrix is a substance composed of ceramides, cholesterol, fatty acids and cholesterol esters. The

percutaneous penetration of exogenous substances varies in the different parts of the body due to the differences in the thickness of the stratum corneum, the highest penetration is in through the thinnest parts of the stratum corneum. A larger variability can be observed between individuals.

Retinoids and their importance in skin physiology

Vitamin A and its derivatives (retinoids) play an important role in skin physiology. Retinoids regulate several effects in differentiation, proliferation, apoptosis, immune regulation, epidermal barrier properties and sensorial functions in numerous skin cell types. Several skin diseases like psoriasis, ichthyosis, skin cancers, acne and various other dermatoses are related to alterations in retinoid metabolism / signaling. Retinoid-based treatments have been shown to be beneficial for various therapeutic approaches of these skin diseases. Topical and systemic treatments with retinoids like, retinoic acid and synthetic retinoic acid analogues or derivatives which modify retinoid metabolism are used already in therapy.

Ingested retinylesters via the diet are hydrolyzed to retinol (ROL) by enteral hydrolases in the intestine. ROL and carotenoids are absorbed by intestinal mucosa cells. Skin expresses lecithin:retinol acyltransferase (LRAT), known to catalyze retinyl-ester synthesis. After intestinal absorption, retinoids can be produced by two pathways: first, retinal (RAL) can be synthesized by oxidative cleavage of the central double bound and this step is followed by the reduction to ROL. ROL binds to cellular retinol binding proteins (CRBP1). Biosynthesis of retinoic acid (RA) from ROL can occur in two steps. ROL is oxidized to RAL by members of the alcohol dehydrogenase (ADH) and retinol dehydrogenases (RDH) through a reversible interconversion; and RAL is further oxidized to RA by means of retinaldehyde dehydrogenase enzymes (RALDH) and cytochrome P450 enzyme family members. The intra-cellular transportation of RA is done by means of cellular retinoic acid binding proteins 1 and 2 (CRABP1, CRABP2).

Polyunsaturated fatty acids, their dietary sources and physiological functions. PUFA metabolism

Dietary fat is an important macronutrient for the growth and development of all organisms. Besides having a nutrient value and its effect on the membrane lipid composition, dietary fat has a strong effect on gene expression as well, which results in changes of metabolism Furthermore, polyunsaturated fatty acids (PUFAs) modulate immune responses, too, thereby showing beneficial effects in a variety of inflammatory disorders.

The 18-carbon long n-6 and n-3 PUFAs are synthesized by several plants, therefore they are obtained from vegetable oils, such as sun-flower oil. However, longer chain members of fatty acids are either obtained directly from animal or marine sources - such as fish or marine oils -, or they are biosynthesized in vivo after the dietary ingestion of the shorter 18-carbon precursors. Various plant oils, such as sunflower or soybean oil, and meat products, contain mainly n-6 PUFAs, such as linoleic acid (LA) (18:2n-6) and arachidonic acid (AA) (18:3n-6), while fish oil mainly contains n-3 PUFAs: eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3).

PUFAs have several physiological functions in the mammalian organism. As mentioned before they are important dietary sources, being essential macronutrients for the growth and development of all organisms. PUFAs are structural components of the cells and they also exert dramatic effects on gene expression by regulating the activity of four families of transcription factors. Moreover, PUFAs play an important role in the skin, contributing to the physical structure of the epidermal barrier, exerting profound effects on the endogenous epidermal phospholipid fatty acid composition and also take part in the generation of anti-inflammatory and antiproliferative metabolites, modulating thus immune responses.

PUFA metabolism involves the alternating Δ6-desaturation, elongation and Δ5-desaturation. Thus gamma-linolenic acid (GLA) is desaturated by FADS2 (fatty acid desaturase 2) from LA which is further elongated to dihomo-gamma-linolenic acid (DHGLA) by fatty acid elongase (ELOVL) 5 and metabolized to AA acid by fatty acid desaturase 1 (FADS1). Similarly, docosapentaenoic acid (DPA) is formed by elongation from EPA and after an intermediate desaturation step, DHA is formed.

Nuclear hormone receptors and retinoids

Retinoic acid activities are mainly mediated by retinoic acid bound to its RA nuclear receptors, the retinoic acid receptor (RAR) family and the retinoid X receptor (RXR) family. It has been demonstrated that RA, bound to an RAR can bind to RA response elements (RAREs) in the DNA and such modulate the transcription of specific genes. These changes in the gene expression are responsible for the biological and also for the therapeutic effects of retinoids.

In humans three distinct RARs have been identified: RARα, RARβ and RARγ. The three RAR genes have different expression patterns in different cells and also during development. RARα gene transcripts are found in most embryonic and adult tissues and may be therefore

responsible for the effects of retinoids on growth and differentiation. RARβ is just expressed in a low level in adult human skin. Most of the RAR receptors in adult skin are RARγ.

The RXR family has also three receptors: RXRα, RXRβ and RXRγ. These receptors have similarities, but are quite different from the RAR receptors, both in structure and ligand specificity. RXRα receptor appears to be the mostly expressed in human epidermis.

Nuclear hormone receptors and fatty acids

Although 9-cis retinoic acid has been described to be the natural ligand for RXR, recently evidence indicates, that it can also become activated by naturally occurring polyunsaturated fatty acids, including docosahexaenoic acid (DHA), arachidonic acid or phytanic acid.

Certain fatty acids and their oxidation products are capable of binding and activating peroxisome proliferators activated receptors (PPARs). It has been shown that the oxidative metabolism of linoleic acid produces several bioactive metabolites that bind to PPAR. A search for natural ligands in human serum identified palmitic acid, oleic acid, linoleic acid and arachidonic acid as endogenous activators of rat PPARα. Unlike the PPARα subtype, PPARγ has a clear preference for polyunsaturated fatty acids. The essential fatty acids linoleic acid, linolenic acid, arachidonic acid and eicosapentaenoic acid have been shown to bind to PPARγ at micromolar concentrations. PPARβ/δ is also a receptor for naturally occurring poly-unsatureated fatty acids, such as dihomo-γ-linolenic acid, arachidonic acid and EPA with low affinities in low micromolar range.

Aim of the studies

We aimed at investigating the gene expression profiles of all known genes involved in retinoid metabolism, namely metabolizing enzymes, retinoid transport proteins, retinoid receptors and target genes and to find out whether retinoid concentrations change in skin and serum of atopic dermatitis patients in comparison to healthy volunteers. In addition our interest was to find out how allergic sensitization, DHA-enriched fish oil supplementation and allergic sensitization with concomitantly DHA-enriched fish oil supplementation of mice influences the percentile distribution of cholesteryl-, triacylglycererol- and phospholipid-esters of the fatty acids and various ratios of eicosanoid precursor PUFA like EPA, DHA and AA in serum.

MATERIALS AND METHODS

Skin biopsies and serum samples

Punch biopsy specimens were taken from involved skin of 6 patients with atopic dermatitis (2 male and 4 female patients; average age 31 years) and from 6 non-atopic healthy volunteers (2 male and 4 female individuals; average age 30 years), who were characterized by the absence of personal or family history of atopic disease. Prior informed consent was obtained according to the guidelines of the Ethical Committee and the declaration of Helsinki. In case of AD patients, one biopsy of affected skin and one biopsy of non-affected skin were taken from each patient. Epidermis represents ca. 20-30 % of the skin biopsy used for analysis. Specimens were immediately frozen in dry ice and stored at -70 °C until RNA isolation or HPLC analysis was performed. At the same time, serum samples were drawn from the same patients and were kept at -70°C until analysis.

Analysis of mRNA expression

Skin samples were homogenized in Tri® reagent solution and total RNA was isolated according to the manufacturer's guidelines. The concentration and purity of RNA were measured by means of NanoDrop spectrophotometer (Thermo) and its quality was checked using agarose-gel-electrophoresis. For real-time quantitative PCR (QRT-PCR), total RNA was reverse transcribed into cDNA using the Super Script II First-Strand Synthesis System (Invitrogen). QRT-PCR was carried out in triplicate using pre-designed MGB assays ordered from Applied Biosystems, on an ABI Prism 7900. Relative mRNA levels were calculated using either the comparative C_T method and were normalized to cyclophilin A mRNA. Sequence Detector Software (version 2.1) was utilized for data analysis.

High performance liquid chromatography mass spectrometry – mass spectrometry (HPLC MS-MS) analysis

Concentrations of retinol and retinoic acids (Ras) were determined in human serum and skin biopsies by our HPLC-MS-MS method. In summary, 100 mg of the skin biopsy (if samples were under 100 mg water was added up to the used standard weight: 100 mg) or 100 µl serum was diluted with a threefold volume of isopropanol, the tissues were minced by scissors, vortexed for 10 seconds, put in a ultra sonic bath for 5 minutes, shaken for 6 minutes and centrifuged at 13000

rpm in a Heraeus BIOFUGE Fresco at +4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 µl of methanol, vortexed, shaked, diluted with 40 µl of 60 mM aqueous ammonium acetate solution and transferred into the autosampler and subsequently analysed.

Statistics

Data are shown as mean and standard error mean values of three measurements per data point. Statistical analysis was performed using the program SPSS 16.0. A *p* value of less than 0.05 was considered significant.

Animal experiments

A set of the animal experiments were performed in the facilities of the Max Rubner Laboratory of the German Institute of Human Nutrition (DIfE) in Potsdam-Rehbrücke, Germany. The experiment was approved by the respective ethical authorities from the Land Brandenburg. A second set of experiments, for investigation of the influence of aluminium hydroxide ($Al(OH)_3$) on lipid distribution, was performed in the Laboratory Animal Core Facility of the University of Debrecen, Hungary. The experiments were performed according to the Hungarian ethical guidelines.

10-12 week old adult female C57BL/6 mice were purchased from Charles River, Sulzfeld, Germany. Mice were kept under controlled conditions at room temperature (21 ± 1°C), constant relative humidity (55 ± 5%) and 12 hour light / dark cycle with light between 6:00 AM and 6:00 PM. Mice had access to food and water *ad libitum*. The composition of the basal diet was 20% casein, 8% sucrose, 50% wheat starch, 10% sunflower oil, 5% cellulose, 5% mineral mix (Mineral-Spurenelemente-Vormischung C1000, Altromin, Lage, Germany) and 2% vitamin mix (Vitamin-Vormischung C1000 Altromin, Lage, Germany). In the diet supplemented with DHA-enriched fish oil, 40% of the sunflower oil was substituted by DHA-enriched fish oil (DHA 500TG SR from tuna) from Croda GmbH, Nettetal, Germany yielding a mass content of 2% DHA and 0,12 % EPA in the animals diet.

The basal diet and the DHA-supplemented diet groups contained each n=12 mice, for the second experiment using $Al(OH)_3$ treatment for basal and DHA-supplemented diet fed groups n=6 mice per group were used.

Before supplementation with basal- and DHA-enriched-fish oil-diet the mice were fed with standard chow diet from Sniff (Soest, Germany) and for the second set of experiments using $Al(OH)_3$ treatment the mice were fed with standard mouse diet from Altromin (Lage, Germany). One week after the starting of the supplementation with the basal- and the DHA-enriched fish oil diet, 6 mice per group were sensitised with ovalbumin (OVA) and 6 mice were treated with phosphate buffered saline (PBS). In the second set of experiments just $Al(OH)_3$ treatment was performed in a comparable manner. Each group was treated either with three intraperitoneal injections of OVA or PBS (or $Al(OH)_3$ in PBS) after a time interval of one week. OVA injections were made according to the protocol previously described by Rühl et al; briefly, 10 µg of OVA absorbed to 1.5 mg $Al(OH)_3$ in 100 µl PBS was given to each animal. The mice were killed by decapitation four weeks after the dietary and three weeks after the OVA/PBS treatments. At 20-30 min after the collection the blood was centrifuged at 1300 g for 3 minutes and serum was obtained. Serum was stored at – 80°C until analysis.

Fatty acid analysis

The fatty acid analysis was performed by our collaborators Tamás Décsi and Tamás Marosvölgyi. For the analysis of plasma fatty acid profiles, frozen plasma samples were thawed, and then the three internal standards (dipentadecanoylphosphatidylcholine-, cholesteryl-pentadecanoate and -tripentadecanoate) were added. Lipids were extracted by the addition of 3 ml chloroform and 1 ml methanol according to the method of Folch et al. The mixture was vortexed at 2000 rpm for 10 min, and then the lower layer was aspirated into vials and evaporated under nitrogen stream. Lipid extracts were reconstituted in 70 µl chloroform and lipid classes were separated by thin layer chromatography. The solvents for thin layer chromatography of plasma lipids were as follows: hexane : diethylether : chloroform : acetic acid (21 : 6 : 3 : 1, by vol.) followed by chloroform : methanol : water (65 : 25 : 4, by vol.). The bands were stained with dichlorofluorescein, removed by scraping and transesterified in 3 N HCl-methanol solution at 84 °C for 45 min.

Fatty acids were analysed by high-resolution capillary gas chromatography using a Finnigan 9001 gas chromatograph (Finnigan/Tremetrics Inc., Austin, TX, USA) with split injection (ratio

1:25), automatic sampler (A200SE, CTC Analytic, Switzerland) and flame ionisation detector with a DB-23 cyanopropyl column of 40 m length (J & W Scientific, Folsom, CA, USA). The temperature program was the following: temperature of injector at 80 °C for 0.1 min, temperature increase by 180 °C/min up to 280 °C, temperature of detector at 280 °C, temperature of column area at 60 °C for 0.2 min, temperature increase by 40 °C/min up to 180 °C, 5 min isothermal period, temperature increase by 1.5 °C/min up to 200 °C, 8.5-min isothermal period, temperature increase by 40 °C/min up to 240 °C and 13 min isothermal period. The constant linear velocity was 0.3 m/sec (referred to 100 °C). Peak identification was verified by comparison with authentic standards. Fatty acid results were expressed as percentages (weight by weight) of fatty acids detected with a chain length between twelve and twenty-four carbon atoms.

Enzyme-linked immunosorbent assay ELISA analysis

The ELISA analysis was performed by our collaborators Margitta Worm and Christine Weise (Koch). Plates were coated overnight with anti-mouse EM95.3 (5 µg/ml; all monoclonal antibodies used were kindly provided by Dr. Lamer, MPI, Freiburg, Germany) diluted in 0.1 M bicarbonate buffer. After blocking with 3 % milk powder/PBS, sera (diluted in 1 % milk powder/PBS) were incubated overnight and were detected with biotin conjugated anti-mouse 84 1-C (1 µg/ml). The reaction was developed with streptavidin peroxidase and tetramethylbenzidine (both from Sigma, Dreieich, Germany) and was stopped with 1 M sulphuric acid. The plates were measured at 450/490 nm and the amount of total IgE was calculated according to the standard curve. The validity was assessed by using a standard with known concentrations for total IgE and by determination of 50% saturation for OVA-specific IgE ELISA. Furthermore, all sera were measured in serial dilutions.

Statistics

Standard deviations have been calculated with SPSS 15.0 (SPSS Inc., Chicago, USA) software for Windows using a two-factor independent Mann-Whitney test; a value of $P<0.05$ was used to determine statistical significance.

RESULTS

Expression profiles of retinoid signalling pathways in AD skin samples

Firstly we investigated the expression profile of genes involved in retinoid-homeostasis, -regulation, - metabolism, retinoid receptors and retinoid-target genes. These data show that a severe dysregulation of retinoid-homeostasis, -metabolism and -signalling is present in affected and non-affected AD skin. The mRNA expression levels were determined by means of QRT-PCR in non-affected and affected skin of atopic dermatitis patients in comparison to healthy volunteers.

Dysregulation of retinol homeostasis / retinyl ester synthesis regulation

Retinol binding protein (RBP) 4 is the carrier protein involved in the transport of retinol and its mRNA expression was non-significantly down-regulated in atopic dermatitis non-affected and affected skin. The mRNA expression of diacylglycerol acyltransferase (DGAT) showed a slight decrease in both affected and non-affected skin compared to healthy skin, while lecithin retinol acyltransferase (LRAT) was significantly up-regulated in non-affected skin and no significant alteration was observed in affected skin of atopic dermatitis patients.

Dysregulation of retinal synthesis

Expression levels of beta - carotene 15,15`- monooxygenase (BCMO1) and beta-carotene-monooxygenase-2 (BCMO2) mRNA were not significantly altered in skin with atopic dermatitis compared to healthy skin. Retinol dehydrogenases and alcohol dehydrogenases are enzymes which are responsible for converting retinol to retinal. Retinol dehydrogenase (RDH) 2, RDH10 and RDH16 showed a very similar pattern in their mRNA expression. RDH2 was statistically significantly augmented in both affected and non-affected atopic dermatitis skin, compared to healthy skin. Induced mRNA expression of RDH10 and RDH16 was observed in atopic dermatitis affected skin, vs. healthy skin. The mRNA expression of alcohol dehydrogenase (ADH) 1C was significantly reduced in non-affected and affected AD skin. CRBP 1 is the intracellular carrier of retinol and its mRNA expression showed a significant down-regulation in case of skin with atopic dermatitis, both in non-affected and affected AD skin.

Reduced retinoic acid synthesis in affected and non-affected skin of atopic dermatitis

Retinal dehydrogenases (RALDH) or acetaldehyde dehydrogenases convert retinaldehyde to RA. We observed a significant decrease of RALDH1 mRNA levels in skin with atopic disease, both in affected and non-affected skin, while the mRNA expression of RALDH2 and RALDH3 did not show significant alterations in our experimental setup. CRABP2 is an intracellular retinoic acid transporter protein and no alteration was observed in its mRNA expression in skin of atopic dermatitis patients. Also CRABP1 did not show a significant alteration in diseased skin, in comparison to healthy skin.

Reduced expression of RA degradation / metabolism enzymes in affected as well as non-affected skin of atopic dermatitis

mRNA expression level of RA-degrading enzymes was determined and revealed a significant decrease in case of CYP26A1 and CYP2S1, both in affected and non-affected AD skin. However mRNA expression of CYP26B1 could not be detected in our experimental setup.

Increased expression of RXRα in non-affected skin of atopic dermatitis

RARα mRNA levels did not show any significant alteration in diseased compared to healthy skin, while mRNA expression of RARβ was slightly but non-significantly up-regulated in non-affected AD skin. By contrast, the expression of RARγ was comparable between atopic dermatitis and healthy skin. The expression of RXRα was significantly increased in non-affected AD skin and affected AD skin.

Expression of retinoid target genes in atopic dermatitis

The mRNA expression of retinoic acid receptor responder (RARRES1) was significantly decreased in case of atopic dermatitis in non-affected and affected skin, in comparison to healthy skin. Also TGM2 (transglutaminase 2) showed a significant decrease both in non-affected and in affected skin of atopic dermatitis. By contrast, HB-EGF (heparin binding EGF-like growth factor) mRNA expression was comparable between AD patients and healthy volunteers.

Due to strongly reduced retinoid signalling pathways in AD skin we next analysed ATRA and ROL concentrations in skin biopsies and serum of healthy volunteers and diseased patients:

Reduced concentrations of retinol and all-*trans* retinoic acid in AD skin, but not in serum of AD patients

Skin concentrations of ATRA and ROL were strongly reduced in affected (ATRA 0.4 / 0.5 ng/g; ROL 37 / 46 ng/g) but also in non-affected skin biopsies (ATRA 0.3 / 0.6 ng/g; ROL 32 / 54 ng/g) from an AD patient in comparison to ATRA and ROL concentrations in healthy skin (ATRA 0.7 / 1.2 ng/g; ROL 207 / 253 ng/g).

Serum concentrations of ATRA 2,8 ± 0,8 ng/ml and ROL 510 ± 217 ng/ml were comparable in healthy volunteers and AD patients ATRA 2,9 ± 1,0 ng/ml and ROL 573 ± 191 ng/ml. No significant differences could be observed between healthy volunteers and AD patients.

Fatty acid composition of serum lipid classes in mice following allergic sensitization with or without dietary docosahexaenoic acid-enriched fish oil substitution

The effect of the different diets and treatments are discussed separately: the effect of diet (basal diet (control diet; CTRL) and PBS injections (CTRL-PBS) vs. DHA-enriched fish oil-supplementation and PBS injections (DHA-PBS); basal diet and OVA injections (CTRL-OVA) vs. DHA-enriched fish oil supplementation and OVA injections (DHA-OVA)); respectively the effect of allergic sensitization without dietary supplementation (basal diet with PBS injections (CTRL-PBS) vs. basal diet with OVA injections (CTRL-OVA)) and with dietary DHA supplementation (DHA-enriched fish oil supplementation with PBS injections (DHA-PBS) vs. DHA-enriched fish oil supplementation with OVA injections (DHA-OVA)).

In a second set of experiments we treated both the animals fed the basal diet and the animals fed the DHA enriched fish oil supplementation with $Al(OH)_3$.

The effect of dietary docosahexaenoic acid (DHA)-enriched fish oil-supplementation *without* allergic sensitization - control diet and phosphate-buffered saline treatment (CTRL-PBS) vs. docosahexaenoic acid (DHA)-enriched diet and phosphate-buffered saline treatment (DHA-PBS)

The sum of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) were comparable between both groups, except for higher values of MUFAs in triacylglycerols (TAG)

in the control animals. *n*-3 PUFA levels were strongly increased after DHA-enriched fish oil supplementation in all the three lipid classes. Inconsistent results were seen in the individual *n*-6 PUFAs. Values of linoleic acid (LA) increased significantly in cholesteryl esters, but decreased in TAG. Similarly, γ-linolenic acid (GLA) values were higher in phospholipids, but significantly lower in TAG and cholesteryl esters in the animals treated with *n*-3 PUFAs in comparison to the controls. Values of DHGLA were significantly higher in both cholesteryl esters and phospholipids in animals treated with n-3 PUFAs than in control mice. In contrast, percentages (w/w) of arachidonic acid (AA) were significantly lower in all three serum lipid classes of animals receiving *n*-3 PUFA supplementation compared to the controls. The sum of *n*-6 PUFAs was always lower in animals receiving *n*-3 PUFA supplementation.

Effect of dietary DHA-enriched fish oil-supplementation with allergic sensitization (CTRL-OVA versus DHA-OVA)

Values of the relevant *n*-3 PUFAs were all significantly higher in the animals supplemented with DHA and EPA. The sums of SFAs were significantly higher in all serum lipid classes in the animals receiving *n*-3 PUFA supplementation. The sums of MUFAs were higher in phospholipids and cholesteryl esters, but were significantly lower in TAG in the animals treated with *n*-3 PUFAs than in the controls. Values of LA were significantly higher in cholesteryl esters, but significantly lower in TAG in the animals receiving DHA and EPA supplementation. AA values as well as the sum of *n*-6 PUFAs were significantly lower in all serum lipid classes in the animals treated with *n*-3 PUFAs than in the controls. In general, all *n*-3 PUFA levels were higher after DHA-supplementation.

Effect of allergic-sensitization without dietary DHA-enriched fish oil-supplementation: control diet and phosphate-buffered saline treatment vs. control diet and ovalbumin treatment (CTRL-PBS vs. CTRL-OVA)

The sums of SFAs were comparable in phospholipids and triacylglycerols in sensitized and non-sensitized animals, while in cholesteryl esters they were significantly lower in the sensitised animals. The sums of MUFAs were similar in phospholipids, triacylglycerols and cholesteryl esters. Allergic sensitization did not cause significant changes in *n*-6 PUFA values. *n*-3 PUFA values exhibited significant changes upon sensitization mainly in triacylglycerols: values of ALA,

and DHA significantly increased, whereas those of EPA and docosapentaenoic acid (22:5*n*-3) significantly decreased. EPA was significantly lower in triacylglycerols and phospholipids and borderline significantly lower in cholesteryl esters.

Effect of allergic-sensitization with dietary DHA-enriched fish oil-supplementation: docosahexaenoic acid-enriched diet and phosphate buffered saline treatment vs. docosahexaenoic acid-enriched diet and ovalbumin treatment (DHA-PBS vs. DHA-OVA).

Sensitization with ovalbumin with dietary interventions resulted in similar SFA levels in cholesteryl esters, but significantly increased levels in triacylglycerols and phospholipids. The sums of MUFAs were similar in all lipid classes. *n*-6 PUFA values were similar in cholesteryl esters and triacylglycerols, while in phospholipids the sum of *n*-6 PUFAs and LA levels were significantly lower in the sensitised animals.

Inter-comparison of lipid classes

DHA-supplementation affected the fatty acid compositions of all three lipid classes vs. control. The increase in the sum of n-3 PUFAs expressed as DHA-PBS/CTRL-PBS was the most pronounced in serum triacylglycerols (30-times), in comparison to phospholipids (4-times) and cholesteryl esters (6-times).

Ratios of EPA/AA, EPA/DHGLA and EPA/DHA

The ratios in the PBS-treated animals were always set as 100% for better comparison of the OVA sensitization values after basal- or DHA-enriched fish oil supplementation diet. The EPA/AA ratio for triacylglycerols descreased significantly and just non-significantly in cholesteryl esters and phospholipids in non-supplemented animals to 23 ± 26%, 29 ± 6%, 29 ± 15% after allergic sensitization, whereas the decrease was only to 70 ± 31%, 80 ± 18%, 76 ± 17% after accompanying DHA-enriched fish oil-supplementation.

The EPA/DHGLA ratio also decreased in non-supplemented animals to 20 ± 18% for triacylglycerols (significantly), 42 ± 15% for cholesteryl esters and 36 ± 22% for phospholipids after allergic sensitization, whereas the decrease was much smaller (to 92 ± 51% for triacylglycerols, 66 ± 26% for cholesteryl esters and 68 ± 13% for phospholipids) after DHA-enriched fish oil-supplementation.

The EPA vs. DHA ratio decreased after sensitization without DHA-enriched fish oil-supplementation to 3 ± 4% significantly for triacylglycerols, 26 ± 6% for cholesteryl esters and 19 ± 9% for phospholipids of the original value, while after DHA-enriched fish oil-supplementation it was significantly higher after sensitization with 119 ± 11% for triacylglycerols, 88 ± 17% for cholesteryl esters and 93 ± 26% for phospholipids.

Influence of $Al(OH)_3$-treatment on the fatty acid composition of plasma phospholipids

Concentrations of AA, DHGLA, DHA and EPA were not significantly altered after $Al(OH)_3$ or $Al(OH)_3$-OVA treatments in basal diet as well in *n*-3-PUFA supplemented animals. Concentrations of EPA were always lower (non-significantly) in OVA- $Al(OH)_3$-treated mice in comparison to PBS-treated mice.

Ratios of EPA/AA, EPA/DHGLA and EPA/DHA were in the same range after $Al(OH)_3$-treatment like for animals with PBS-treatment in basal diet fed animals, while the ratios were much, but non-significantly, lower in OVA-$Al(OH)_3$-treated animals. Ratios of EPA/AA, EPA/DHGLA and EPA/DHA were just slightly lower after $Al(OH)_3$-treatment, ratios of EPA/AA and EPA/DHA were lower and just EPA/DHGLA ratios were significantly lower in the *n*-3 PUFA diet supplemented mice.

n-3 PUFA supplementation diet significantly ameliorated the decrease of the EPA/AA, EPA/DHGLA and EPA/DHA ratios in plasma phospholipids after OVA-$Al(OH)_3$-treatment but not after $Al(OH)_3$-treatment alone in comparison to basal diet fed animals.

Allergic sensitization

Serum IgE levels increased significantly after OVA sensitization in the control diet fed group from 0.4 ± 0.2 μg/ml to 3.2 ± 2.0 μg/ml and in the DHA-enriched fish oil supplemented group from 0.7 ± 0.1 μg/ml to 2.8 ± 1.3 μg/ml. There was no significant difference between the IgE levels of the control diet fed group and the DHA-enriched fish oil supplemented group. Additionally the specificity of the sensitization was supported by measuring OVA-specific IgE titers in sensitized mice whereas in non-sensitized mice no OVA-specific IgE was detected.

DISCUSSION

Retinoids are known to regulate several events relevant for skin diseases such as epidermal differentiation, proliferation, apoptosis, immune regulation and epidermal barrier properties. In previous studies it has been shown that retinoid supplementation evokes high IgE levels and alters Th1/Th2 balance. Several retinoids, such as retinoic acid and synthetic retinoic acid analogues have been used therapeutically in skin and immune diseases and proved to be beneficial. Atopic dermatitis is a chronically relapsing inflammatory skin disorder, characterized by disturbed epidermal barrier function, an increased Th2 immune response and elevated IgE levels. So far the expression profiles of the genes involved in retinoid homeostasis, regulation and metabolism, retinoid receptors and target genes have not been investigated in skin of patients with atopic dermatitis in comparison to healthy volunteers.

De**creased retinoid concentration and retinoid signaling pathways in human atopic dermatitis**

In this study we demonstrated that in affected and in non-affected human tissue biopsies the retinoid transport, synthesis, concentrations, signaling and homeostasis are severely dysregulated in comparison to skin from healthy volunteers. Surprisingly even the skin of non-affected areas of atopic dermatitis patients displayed dysfunction of retinoid signaling, suggesting an intrinsic disease specific dysfunction for the regulation of retinoid binding proteins, metabolizing enzymes, retinoid response target genes expression as well as retinoid concentrations. Interestingly the mRNA expression of the majority of retinoid response target genes like CRBP1, CYP26A1, CYP2S1, TGM2 and RARRES1 were significantly down-regulated, which is in accordance with the decreased level of retinoic acid determined in AD skin.

ATRA is the major RAR ligand and its concentration in the mammalian skin is tightly regulated in a specific spatiotemporal. Various cell types in the skin and especially in the inflamed skin have been shown to be able to synthesize the bioactive retinoic acid. We found that retinoid response target genes like RARRES1, CRBP1, CYP26A1, CYP2S1 and TGM2 are significantly decreased in affected as well as non-affected human skin of AD patients, while the expression of other retinoid target genes like RARβ, CRABP2 and HB-EGF were not altered. HPLC MS-MS data additionally confirmed that the concentration of ATRA is much lower in affected and in non-affected AD skin in comparison to skin from healthy volunteers; no

difference was detected between affected and non-affected AD skin. This might also be a cause or result of lower delivery of the retinoic acid precursor, retinol (ROL) via RBP4 to the skin, while both ROL levels as well as RBP4 expression are lower in affected AD skin samples in comparison to healthy volunteers.

Additionally the analysis of serum concentrations from the same patients and volunteers displayed comparable ATRA levels between AD patients and healthy volunteers. These data suggest a systemic non-RA based origin for this skin specific dysfunction of retinoid mediated signaling in AD. We suggest and partly know already that besides ATRA (the main signaling molecule for retinoid target gene expression), which is much lower in skin of atopic patients, also other relevant and still non-identified bioactive retinoids or / and other retinoid mediated response pathways involving retinoid-activated nuclear receptors must be present. Alternative activators of RAR and RXR may be responsible for stable and non-altered expression of the retinoid target genes RARβ, CRABP2 and HB-EGF in atopic skin even when ATRA levels are present in much lower concentrations. Identification of novel endogenous RAR as well as RXR ligands is under investigation in our laboratories.

The expression of the major retinoic acid synthesizing enzyme in the skin the RALDH1 is significantly decreased in AD skin vs. healthy skin. This strong down-regulation in affected and non-affected AD skin is suggested to be mainly responsible for lower ATRA concentrations and thereby for the significantly lower retinoid mediated signaling in the skin of AD patients.

Deficiency of retinoids / retinoid signaling in the skin or general vitamin A deficiency has been associated to various symptoms also seen in the atopic dermatitis skin phenotype. Th1 / Th2 shift, altered apoptosis, altered skin differentiation and proliferation and increased bacterial skin colonization were associated with vitamin A deficiency or deletion of retinoid receptor mediated signaling in transgenic skin specific mouse models. Whether lower retinoid signaling and lower retinoic acid concentration in AD skin is based on an intrinsic abnormality it is still under examination in various *in vivo* studies in our laboratories.

Remarkable is the reduced gene expression of the retinoid-target genes CRBP1, CYP26A1, CYP2S1, TGM2, RALDH1, RARRES1 and the ADH1C in non-affected AD skin comparably to affected AD skin. We suggest that a general and intrinsic abnormality is responsible for this dysregulation and maybe a result of systemic chronic inflammation. A different expression profile

was observed for LRAT and RXRα which are exclusively increased in non-affected AD skin, confirming also a general intrinsic abnormality responsible for this dysregulation of retinoid-signaling (LRAT, RXRα) and maybe of other RXRα-heterodimer mediated pathways in non-affected AD skin. This increased expression of LRAT and RXRα maybe a response of the non-affected skin on intrinsic chronic inflammation to further enable and balance reduced retinoid signaling. Additionally the increased expression of RDH2 and RDH10 may be a skin based response to enable and balance retinoid signaling in the skin.

An altered nutrition with high vitamin A as well as pro-vitamin A carotenoids resulting in significantly higher serum levels of all-*trans* retinoic acid or increased ingestion of dietary fats which lead to increased expression of various factors / enzymes important for retinoid signaling might contribute also to this altered retinoid signaling in affected as well as non-affected skin of AD patients.

Several approaches using nutritional supplementations with carotenoids and various retinoids as well as systemic inflammation / allergic sensitization are in progress to elucidate why both in affected as well as in non-affected skin of atopic dermatitis patients retinoid transport, synthesis, concentrations and signaling are strongly decreased. We suggest that the answer to this question may help to understand the pathogenesis of atopic dermatitis and may lead to strategies for atopy prevention. Based on our observations we suggest that topical retinoid applications using single retinoids or combinations of some selective retinoids would be highly beneficial for atopic dermatitis therapy.

In summary, more studies are needed to identify how retinoid transport, metabolism, concentrations and signaling are regulated in the skin and the regulation of key players like RALDH1, which is the major enzyme important for retinoic acid synthesis in human skin, in AD patients in comparison to healthy volunteers. Animal studies using topical as well as systemic application of various retinoids and KO animal models of retinoid synthesizing enzymes and retinoid receptors are in progress. We conclude that the retinoid signaling pathway is dysregulated in AD patients based on an abnormal retinoid transport, synthesis and concentrations which might contribute to the pathogenesis of AD, but also offer novel therapeutic approaches.

In our study we have shown that retinoid signaling pathways and retinoic acid levels are lower in skin of AD patients in comparison to healthy volunteers, additionally, other studies have shown

significant changes of the PUFA composition in the skin and serum of atopic dermatitis patients. On the other hand, retinoid supplementation studies have shown to evoke high IgE levels and alter Th1/Th2 balance. We aimed to investigate the impact of allergic sensitization on the PUFA composition of various serum lipid classes as well as IgE secretion in a mouse model with or without dietary DHA-enriched fish oil supplementation.

Fatty acid composition of serum lipid classes in mice following allergic sensitization with or without dietary docosahexaenoic acid-enriched fish oil substitution

In this study, we demonstrate that both DHA-enriched fish oil-supplementation and allergic sensitization significantly influence the fatty acid composition of different serum lipid classes. Allergic sensitization after three intra-peritoneal injections of OVA associated to $Al(OH)_3$ has been shown in various publications of our group and additionally in this study we determined an increased total IgE level after OVA sensitization. The levels of IgE were just slightly lower in the DHA-enriched fish oil-supplemented group in comparison to the control diet fed group. The fact that the alteration of ratios of individual fatty acids being precursors of pro-inflammatory or anti-inflammatory PUFA-metabolites changed after allergic sensitization in a manner depending on DHA-enriched fish oil-supplementation is firstly described in literature.

Supplementation of DHA has been shown in several studies carried out in various organisms to yield in increased concentrations of DHA as well as EPA in serum lipids. Our data support this aspect of previous reports in a mouse model investigating the fatty acid composition of serum triacylglycerols, phospholipids and cholesteryl esters. The effect of DHA-enriched fish oil-supplementation on EPA levels could be mediated via three pathways: a) Increased concentrations of EPA in the DHA-enriched fish oil, b) *retro*-conversion of DHA to EPA and c) decreased conversion of EPA to DHA. In addition to the increased levels of the main n-3 long-chain PUFAs, DHA and EPA, the levels of the n-6 long chain PUFA, AA markedly decreased. This finding is in accordance with previous observations, and may reflect the inhibitory effects of abundance of DHA on delta-6-desaturase, the rate limiting step also in AA biosynthesis, based on higher concentrations of AA precursors in sunflower oil in comparison to DHA-enriched fish oil.

The exact interrelationship of fatty acids in circulating serum lipids to tissue fatty acid metabolism remains to be clarified. However, fatty acid composition of serum lipid classes is still considered an important indicator of fatty acid status. In the present study, effects of dietary

intervention were detectable in all the three serum lipid classes analyzed, whereas marked effect of allergic sensitization on n-3 long-chain PUFAs could mainly be observed in serum triacylglycerol esters.

The major outcome of our study was the influence of the allergic sensitization on the fatty acid composition of various serum lipid classes. To the best of our knowledge, no study has previously focused on the effect of allergic sensitization on serum fatty acid patterns, whereas several studies compared fatty acid status in humans with and without allergic disease. Possibly these alterations are partly due to an altered lipoprotein distribution after acute phase response.

In the present study, we investigated the effects of allergic sensitization induced by triple intra-peritoneal OVA-injections in adult mice, and observed several significant alterations of plasma phospholipids fatty acid levels. Our experiments showed that $Al(OH)_3$ has no or just marginal effects on lipid composition. This biochemical inactive $Al(OH)_3$ adjuvant is used to potentate the immune responses to vaccines by adsorbing the antigen. $Al(OH)_3$ does not alter serum lipid concentrations, because if given intra-peritoneally and absorbed to allergen, while aluminium or aluminium ions could be quite active in alteration of lipid metabolism. No relevant amounts of aluminium and aluminium ions will reach in blood circulation in case if biochemical stable $Al(OH)_3$ is used under physiological pH conditions after intra-peritoneal injections.

After allergic sensitization but without accompanying DHA-supplementation, the levels of mainly the n-3 PUFAs, ALA, EPA, docosapentaenoic acid (DPA) and DHA were significantly altered in triacylglycerols. EPA and DPA were significantly down-regulated, whereas DHA and ALA were significantly up-regulated.

Ratios of EPA/AA and EPA/DHGLA were significantly reduced upon allergic sensitization and without accompanying DHA-enriched fish oil-supplementation. After accompanying DHA-enriched fish oil-supplementation, however, the reduction of the ratios was much lower. Consequently, the ratios were significantly lower in the non-supplemented than in supplemented animals.

Metabolism via cycloxygenase (COX) and lipoxygenase (LOX) pathways has been shown to be highly dependent on the availability of lipid-precursors for further metabolism. It has been convincingly demonstrated that n-3 PUFAs were better substrates for the conversion by

lipoxygenases, but much weaker for the COX-2 mediated pathways to PgE derivatives. In addition, leukotriens originating from EPA have been shown to be much less active in comparison to analogues from the AA cascade. EPA and other n-3 PUFAs were also found to inhibit COX and LOX activity.

Our data strongly support the hypothesis that DHA-enriched fish oil-supplementation significantly alters the levels and ratios of n-3/n-6 precursor fatty acids for further bio-activation to pro-inflammatory prostaglandins, which are mainly TH-2 skewing, and leukotriens. Various immune-competent cells like lymphocytes, macrophages, dendritic cells etc. could alter lipid metabolism via various enzymes like lipoxygenases, cycloxygenases, cytochromes and etc. as well as various immune reactions could be influenced by dietary lipids and their active metabolites. Novel studies support that EPA and DHA via COX- and LOX- pathways serve as precursors for anti-inflammatory bioactive lipids like lipoxins, neuroprotectins and resolvins. Higher levels of DHA and EPA may lead to increased production of these novel-described derivatives in the mammalian organism after DHA-enriched fish oil-supplementation and accompanying allergic sensitization, possibly resulting in reduced severity of the allergic phenotype.

In summary, the data obtained in the present study indicate that fatty acid levels and especially the ratios of fatty acid representing precursors of bioactive lipids after allergic sensitization highly depend on accompanying DHA-enriched fish oil-supplementation. This DHA-enriched fish oil-supplementation mediated alteration of lipid ratios of bioactive-precursor lipids may explain the allergy ameliorating effects of DHA in particular and/or n-3 PUFAs in general unfortunately in our experimental setup we could not observe any allergy ameliorating effect. Further studies of our group will focus on lipid metabolism and molecular mechanisms of possible allergy preventive effects of DHA in animal studies, human supplementation trials and human cohort studies.

SUMMARY

Polyunsaturated fatty acids (PUFAs) are present in high concentrations in the human organism and are essential components of various layers of the skin, furthermore they are important precursors of several pro- and anti-inflammatory mediators. Several studies investigated the PUFA composition in atopic patients and healthy volunteers and PUFA levels have been shown to be decreased in atopic dermatitis skin in comparison to healthy skin. Alterations of retinoid metabolism and signaling have been related to several skin diseases including atopic dermatitis (AD), but so far the expression profiles of the genes involved in retinoid homeostasis regulation and metabolism, retinoid receptors and target genes as well as retinoid concentrations have not been investigated in the human skin. In previous studies, in mice, retinoids have been shown to enhance Th2-mediated responses, respectively elevate IgE levels.

Our results show that the target gene expression of retinoid receptor regulated pathways is significantly decreased in AD patients. The main retinoic acid synthesizing enzyme, retinal-dehydrogenase 1, was significantly lower expressed in AD patients. Analysis of retinoid concentrations in serum and skin showed comparable all-*trans* retinoic acid (ATRA) and retinol (ROL) concentrations in AD and healthy serum, but strongly reduced ATRA and ROL concentrations in AD skin in comparison to healthy skin. Our data indicate that retinoid transport, synthesis, concentrations, signaling and homeostasis are severely dysregulated in affected and non-affected human skin biopsies of AD patients in comparison to the skin of healthy volunteers.

In the case of PUFAs the missing point was to find out how supplementation of non-sensitized and sensitized mice with DHA-enriched fish oil diet influences the percentile contribution of n-3 / n-6 PUFAs. Significantly increased percentile contributions of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in case of triacylglycerols, cholesteryl esters and phospholipids, while significantly lower arachidonic acid (AA) levels were observed in the serum of both non-sensitized and sensitized mice fed the DHA-enriched diet. Allergic sensitization was reflected by the decreased EPA/AA ratio in all lipid classes in non-supplemented animals and to a lesser extent DHA-enriched fish oil supplemented mice. Serum IgE levels significantly increased after allergic sensitization both in the group fed the basal diet and the group fed the DHA-enriched fish oil diet, but no significant changes could be observed in between the two diet groups.

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Register Number: DEENKÉTK /38/2011.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Johanna Mihály

Neptun ID: MYH3NK

Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Mihály, J.**, Gamlieli, A., Worm, M., Rühl, R.: Decreased retinoid concentration and retinoid signalling pathways in human atopic dermatitis.
   Exp. Dermatol. accepted by publisher, 5.p., 2011.
   IF:3.239 (2009)

2. Rühl, R., Koch, C., Marosvölgyi, T., **Mihály, J.**, Schweigert, F.J., Worm, M., Décsi, T.: Fatty acid composition of serum lipid classes in mice following allergic sensitisation with or without dietary docosahexaenoic acid-enriched fish oil substitution.
   Br. J. Nutr. 99 (6), 1239-1246, 2008.
   IF:2.764
   DOI: http://dx.doi.org/10.1017/S0007114507862374

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

16 March, 2011

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA EGYETEMI ÉS NEMZETI KÖNYVTÁR

H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

FIRST AUTHORED POSTERS ON INTERNATIONAL MEETINGS

Mihály J, Gamlieli A, Rasche C, Worm M , Rühl R (2010), Dysregulation of retinoid metabolism in human atopic dermatitis, presented at the International Symposium "One hundred years of Ivan Djaja's (Jean Giaja) Belgrade School of Physiology, Belgrade, September 10-14, 2010

Mihály J, Gamlieli A, Rasche C, Worm M , Rühl R (2009), Influence of poly-unsaturated fatty acids and their mono-hydroxylated metabolites on gene expression of nuclear receptor specific target genes, presented on the Workshop "Mitochondria and reproduction", Sofia, Bulgaria, June 1-4, 2010

Mihály J, Gamlieli A, Rasche C, Worm M , Rühl R (2009), Dysregulation of retinoid metabolism in human atopic dermatitis, Journal of Investigative Dermatology 129 (2) presented at the 39th ESDR Meeting 2009, Budapest, Hungary, September 9-12, 2009

Mihály J, Gamlieli A, Rasche C, Worm M , Rühl R (2009), Dysregulation of retinoid metabolism in human atopic dermatitis, presented at "Nuclear Receptor Signaling: From Molecular Mechanisms to Integrative Physiology" Summer School organized by FEBS, Island of Spetses, Greece, August 23 - 28, 2009

Mihály J, Rühl R (2008), Influence of polyunsaturated fatty acids, their mono-hydroxylated metabolites and eicosanoids on gene expression of nuclear receptor specific target genes, Acta Biochimica Polonica, 55 (1), presented at COST Action 926 Conference, Krakow, Poland, 2008

Mihály J, Rühl R (2007), Influence of polyunsaturated fatty acids and their mono-hydroxylated metabolites on gene expression of nuclear receptor specific target genes, Annals Nutr. Metab. 51 (1), 2007, presented at the 10th European Nutrition Conference, Paris, France, 2007

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Ralph Rühl, for the training and fruitful scientific discussions, his ambitions for motivation and for the solution of problems occurring during the conduction of my work.

I am also grateful to professor László Fésüs for an open academic research atmosphere at the Department of Biochemistry and Molecular Biology. His presence was always a guarantee of meaningful discussions.

I am thankful for the working group of Prof. László Nagy for kindly providing materials and instruments. I also thank Szilárd Póliska for his help.

I would like to thank our collaborators Prof. Tamás Décsi and Tamás Marosvölgyi for performing the fatty acid analysis and Prof. Margitta Worm and Christin Weise (Koch) for the ELISA analysis.

I am grateful for my colleagues Gamze Aydemir, Emőke Bartók, Anat Gamlieli, Janine Gericke and Kathrin Weiss. I thank for their support, advices and help with my problems. I am especially indebted for their friendship, their emotional support and for the pleasant working atmosphere. I also thank Vinczíné Éva Papp for her excellent technical assistance.

Last but not least I would like to thank my parents Zsigmond and Erzsébet Mihály, and to my sister, Szendi, for helping me achieve my dreams in life, I am grateful for their unconditional love, patience and support in all situations.