Nutrigenomics in human peripheral blood mononuclear cells

The effects of fatty acids on gene expression profiles of human circulating cells as assessed in human intervention studies

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG
(Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid)

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 20 februari 2009
des namiddags te half twee in de Aula.

Mark Bouwens

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Thesis Wageningen University, Wageningen, the Netherlands, 2009.
With abstract – with references – with summary in Dutch.

ISBN: 978-90-8585-327-5

Abstract

Nutrigenomics in human peripheral blood mononuclear cells. The effects of fatty acids on gene expression profiles of human circulating cells as assessed in human intervention studies

PhD thesis by Mark Bouwens, Division of Human Nutrition, Wageningen University, Wageningen, the Netherlands, February 20, 2009

Peripheral blood mononuclear cells (PBMCs) are circulating immune cells. Gene expression profiles of PBMCs have been increasingly used as diagnostic biomarkers for diseases such as leukemia and multiple sclerosis. PBMCs are relatively easy and less invasively to obtain compared with other human tissues. Although nutrition has been shown to play a pivotal role in the development of diseases such as diabetes mellitus type 2 and the metabolic syndrome, up until now little was known about the effects of nutrition on PBMC gene expression profiles. Nutrients such as polyunsaturated fatty acids (PUFAs) have been shown to have a profound impact on metabolism, through activation of transcription factors such as peroxisome proliferator-activated receptors (PPARs), resulting in numerous changes in gene transcription. This has been mainly demonstrated in various *in vitro* cell and *in vivo* animal models. Since PBMCs express PPARs, they may be very suitable to study the effect of fatty acids on gene expression profiles *in vivo* in the human situation.

In this thesis we examined the effects of several fatty acids on the gene expression profiles of human PBMCs, using whole genome microarray analysis in *ex vivo* incubation experiments and human intervention studies. We demonstrated that fasting, which elevates levels of endogenous free fatty acids in the blood, results in changes in gene expression profiles of PBMCs. These changes in gene expression partially seemed to be mediated through PPARα activation. The function of PPARα in human PBMCs was further explored by means of microarray analysis. We concluded that PBMCs are suitable cells to study the effects of PPAR-medi-

ated processes in healthy humans. In addition, we showed that fatty acids affect PBMC gene expression profiles in healthy humans during a postprandial intervention as well as after a long-term dietary intervention. This resulted in distinct fatty acid specific gene expression profiles, and revealed that long term effects of dietary fatty acids are different from postprandial effects. Moreover, we demonstrated that the application of whole genome PBMC gene expression profiles is a very sensitive method, as we were able to detect effects that were previously only observed in *in vitro* and animal experiments. In summary, we conclude that gene expression profiles of human PBMCs can be used to reflect changes in dietary fatty acid intake. These results provide an encouraging basis for future research in which the utility of PBMC gene expression profiles as preventive diagnostic tools for nutrition-related disorders can be investigated.

Contents

Chapter 1

General introduction

Nutrigenomics

Nutrigenomics is the study of the genome-wide impact of nutrition to understand the subtle associations between nutrition and health[1]. This can be examined on several levels, including the genome, the transcriptome, the proteome and the metabolome. Genomics is the study of an organisms entire genome, transcriptomics is the analysis of the expression levels of all mRNAs in a cell population or an organ, proteomics is the large-scale study of proteins, their structures and interactions and metabolomics examines and profiles the collection of all metabolites in a biological organism. Although a significant number of studies on the genome-wide impact of nutrition and bioactive food components have been performed *in vitro* and in animal models[2, 3], nutrigenomics studies on healthy human subjects are still limited in number.

Transcriptomics

Transcriptomics utilizes high-throughput genomic tools, such as DNA microarrays, to examine expression of thousands of genes, at a given time and under various conditions including environmental (e.g. nutritional) challenges. In this way, patterns of gene expression can be used to get insights in genome-wide effects of a particular stimulus and on the potential involvement of specific regulatory pathways involved in the transcriptional regulation. The use of high-throughput genomic tools has already proven to be extremely valuable in molecular medicine and pathology, where it improved the understanding of the pathogenesis of complex diseases[4] and brought new diagnostic opportunities[5, 6]. Transcriptomics in humans has not only shown to be able to distinguish a pathological state from the normal, non-pathological state, but is also able to provide detailed patterns that reflect different stages in disease progression[7-9]. These features show the potential of gene expression profiles to be predictive for metabolic changes and the usefulness of these profiles as diagnostic tools[10].

Tissue availability

The major disadvantage in transcriptomics studies in healthy humans is the availability of tissue because of the invasiveness of biopsy procedures. Most of the reported human transcriptomics studies have been performed with patients that had to undergo tissue sampling for medical and/or diagnostic purposes. In healthy humans the main options for tissue sampling procedures for nutrige-

nomics studies are muscle and adipose tissue biopsies. However, these are still quite cumbersome and painful procedures. In contrast with muscle and adipose tissue biopsies, blood is a relatively easily obtainable tissue in healthy subjects. However, RNA extraction from whole blood has the disadvantage of a large background of globin RNA, originating from immature erythrocytes. Besides erythrocytes, blood contains thrombocytes, small cytoplasmic bodies derived from cells, and leukocytes. In nearly all mammals the nucleus of erythrocytes has been extruded during the final maturation stage, making them unsuitable for gene expression analysis. Thrombocytes contain only limited amount of RNA and are thus difficult to employ. Leukocytes do not have these limitations and can be found throughout the body, including the peripheral blood and lymphatic system. These cells can be divided in two varieties, mononuclear and polynuclear cells. The peripheral blood mononuclear cells (PBMCs) include the lymphocytes and the monocytes.

Peripheral blood mononuclear cells

PBMCs are relatively easy to isolate from whole blood. Gene expression profiles of PBMCs have been used to distinguish a variety of diseases from the health state, to differentiate between disease stages, and to predict clinical outcome [7-9, 11-16]. Another study identified genes that are differentially expressed in the different cells within PBMCs[17]. Also, variation in gene expression profiles between and within individuals was examined in various studies[17-19], which demonstrated that variation in PBMC gene expression profiles is relatively high between subjects, but far lower within individuals over time.

PBMCs are a critical component in the immune system to fight infection and adapt to invading microorganisms. Lymphocytes are the largest subpopulation within PBMCs and play an important role in the adaptive immune response, whereas monocytes are important cells of the innate immune response. The adaptive immune system recognizes and remembers specific pathogens, which results in stronger attacks each time the pathogen is encountered[20]. The innate immune system provides immediate defense against infections and functions in a non-specific manner.

Lymphocytes

Lymphocytes can be divided into two major groups, the T lymphocytes, which develop in the thymus, and B lymphocytes, which originate from the bone marrow. T lymphocytes are the largest group, and they are involved in cell mediated immunity. B lymphocytes are primarily responsible for the humoral immunity, by producing large quantities of antibodies which neutralize foreign invaders such as bacteria and viruses. T lymphocytes in turn can be split up in helper T (Th) cells, also known as CD4+ lymphocytes, and cytotoxic T (Tc) cells, also known as CD8+ lymphocytes. Th cells produce cytokines, that direct the immune response, whereas Tc cells produces toxic granules that induce the death of pathogen-infected cells[20].

Monocytes

Monocytes play a large role in the innate immune defense[21]. In response to inflammatory signals, monocytes can move rapidly (approx. 8-12 hours) to sites of infection in the tissues and differentiate into macrophages. Macrophages perform phagocytosis to remove foreign substances in the body, by binding to the microbe directly via pattern-recognition receptors or by using intermediary proteins such as antibodies that coat the pathogen. Monocytes are also capable of killing infected host cells via antibodies, similar to Tc cells, termed antibody-mediated cellular cytotoxicity. Macrophages are responsible for protecting tissues from foreign substances, but are also suspected to be the predominant cells involved in the development of atherosclerosis. During atherosclerotic development, macrophages can take up lipids and differentiate in foam cells, which are known as prototypical atherosclerotic cells[22].

Effects of nutrients on gene expression

Nutrients can influence the transcriptional control of gene expression by means of specific transcription factors. A very important group within the nutrient sensing transcription factors are the large subfamily of nuclear receptors, with 48 members. By activation of specific members of this subfamily, nutrients are able to influence a wide variety of cellular functions. An example for this principle are peroxisome proliferator-activated receptors (PPARs), which serve as most important fatty acid sensors in the cells[1].

PPARs

PPARs are ligand-activated transcription factors that bind mainly unsaturated or very long-chain fatty acids and their derivatives, which results in transcriptional activation. Regulation by PPARs requires heterodimerization with the retinoid X receptor (RXR), another nuclear receptor. After activation by ligand binding, the PPAR/RXR heterodimer results in transcription via binding to DNA response elements (PPREs) often present in or near the promoter of target genes (**Figure 1**). This usually results in up-regulation of these genes, but can also result in a repression of transcription by interaction with other transcription factors and repression of their mechanism of action, a process called transrepression[23].

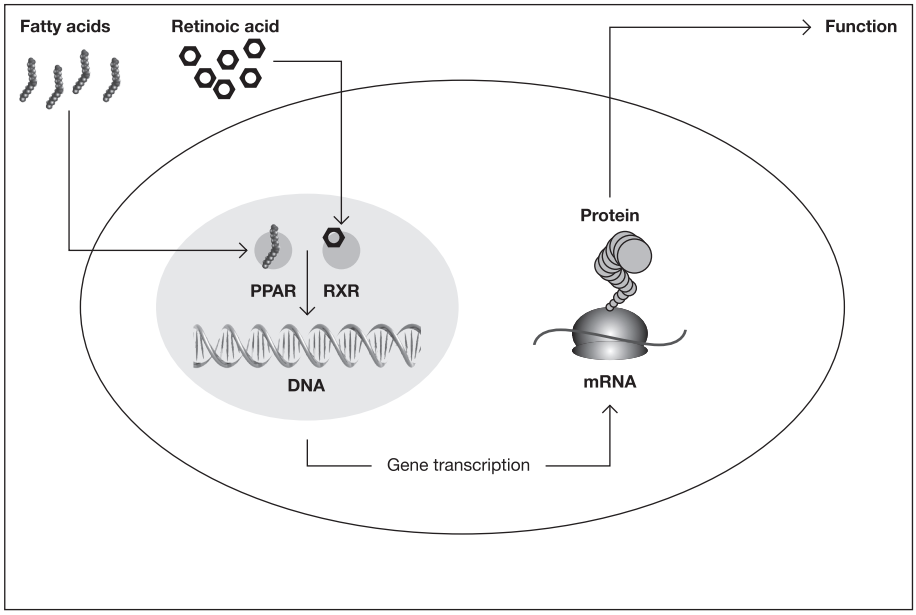

Figure 1 PPAR signaling Ligands, such as unsaturated fatty acids, enter the cell and bind peroxisome proliferator-activated receptors (PPARs). 9-cis retinoic acid binds its heterodimeric partner, the retinoid X receptor (RXR). The PPAR/RXR heterodimer binds to specific response elements (PPREs) located within or near the promoter of PPAR target genes. Binding results in increased expression of these target genes, consequently leading to protein synthesis.

Three types of PPARs have been identified: alpha, delta (beta), and gamma[24]. PPARα is mainly expressed in liver, intestine, kidney, heart, skeletal muscle and adipose tissue. PPARβ/δ is ubiquitously expressed, but markedly present in

brain, adipose tissue and skin. PPARγ is expressed in three forms, through alternative splicing. PPARγ1 is ubiquitously expressed, PPARγ2 is mainly expressed in adipose tissue and PPARγ3 is expressed in macrophages, colon and white adipose tissue. Each of the PPAR subtypes has specific functions, but all three PPARs affect energy homeostasis and inflammatory responses[24].

The activity of PPARs can be modulated by drugs such as the hypolipidemic fibrates (PPARα) and the insulin sensitizing thiazolidinediones (PPARγ), but fatty acids are the natural ligands of these transcription factors. Of all the natural ligands of PPARs, polyunsaturated fatty acids have been shown to activate PPARs most profoundly[25, 26].

Fatty acids

As fatty acids are known to alter gene expression, they are suitable candidate nutrients to study the effects of nutrition on whole genome gene expression. Fatty acids can be grouped by their degree of saturation and length. The main fatty acids in the diet are monounsaturated (MUFA) and saturated fatty acids (SFA). Polyunsaturated fatty acids (PUFAs) are present in the diet in much lower quantities. However, this low intake of PUFAs is relatively important, since the human body can produce most fatty acids it needs, except for two long chain PUFAs. These two, linoleic acid (LA) and alpha-linolenic acid (ALA), are found in plant oils. In addition, the longer-chain n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can only be converted from LA and LNA to some extent. It is therefore advised to consume fat fish on a regular basis, since EPA and DHA are abundantly present in fish oil.

Intake of PUFAs has been suggested to have a variety of beneficial health effects, ranging from prevention of cardiovascular disease[27] and a reduced production of inflammatory agents[28] to decreased cognitive decline[29]. It is still unknown how the beneficial effects of PUFAs on health are mediated. It is, however, well known that these fatty acids are transported though the human body by the blood either largely albumin-bound as free unesterified fatty acids (FFA) or as triglyceride in the lipoprotein fraction. Fatty acids in blood can originate from two sources, either endogenous, from the fatty acid pool stored in adipose tissue, or exogenous, directly from the diet.

During fasting, fatty acids will be released from adipocytes into the bloodstream in the form of FFAs. These endogenous FFAs rise during fasting, and drop after a

regular meal (postprandial). However, after a high fat meal the concentrations of FFA can rise, and the intake of specific fatty acids is reflected in the FFA profile in the blood[30].

Postprandial, short- and medium chain fatty acids can be absorbed directly into the blood, but long chain fatty acids are to large to be directly absorbed and are therefore transported as triglycerides in large lipoprotein particles called chylomicrons, via the lymphatic vessels into the bloodstream. In the blood chylomicrons transport these lipids to peripheral tissues such as adipose, cardiac and skeletal muscle tissue, where their triglyceride components are broken down by the activity of lipoprotein lipase. During a high fat meal an overload of fatty acids enter the system and not all fatty acids that are released from chylomicrons are absorbed in the peripheral tissue, resulting in a rise in blood FFA, postprandially. Besides their elevation during fasting and after a high fat meal, FFA concentrations can also be increased in healthy subjects during long-endurance exercise or in less healthy people with obesity and diabetes mellitus type 2, because of the reduced capacity of peripheral tissues to rapidly remove FFAs.

Nutritional effects on PBMC gene expression profiles

In nutrition-related research we are mainly interested in the prevention of disease, because once the disease is manifested, drug treatment is required and nutritional intervention is of less importance. For that reason nutritional research is performed in relatively healthy people. Nutrients like fatty acids are known for their effects on health, which can be positive, in the case of PUFAs, or less positive, in the case of SFA. How these fatty acids exert their effects on health in mainly unknown, but it is known that fatty acids can influence gene expression by transcription factors such as PPARs. With the use of genomic tools such as transcriptional profiling with DNA microarrays, we can measure all transcriptional regulated processes influenced by fatty acids. In healthy individuals, PBMCs are highly suitable to be used for this purpose, since these cells are easily obtainable, there gene expression profiles have been used to reflect pathological conditions and it has been shown that these cells express the fatty acid sensing transcription factor PPARα[31, 32].

Characterization of nutrient-specific effects on gene expression in humans could advance understanding of the mechanisms involved in nutritional effects on important phenotype features such as cellular metabolism and homeostatic control.

In the end this could lead to evidence-based dietary advice in order to prevent disease-related cellular dysfunction or intervene in a very early state of pathological disarrangements.

Outline of this thesis

We hypothesized that gene expression profiles of peripheral blood mononuclear cells are able to reflect nutritional changes. More specifically, we speculated that PBMC gene expression profiles could show the acute and long-term effects of both endogenous and dietary fatty acids in healthy humans. To test this hypothesis we initially performed a study in which the endogenous fatty acids in plasma of healthy young men were increased by fasting for 48 hours, and changes in gene expression profiles of the PBMCs were assessed by whole genome microarray analysis (**Chapter 2**). To further elucidate the role of PPARα in PBMCs an *ex vivo* incubation study using comprehensive whole genome gene expression analysis was performed. The fatty acid receptor PPARα was activated by incubation of the cells with the synthetic PPARα ligand WY14,643 to show the specific PBMC PPARα response (**Chapter 3**). Subsequent studies were performed to reveal dietary effects of fatty acids on PBMC gene expression profiles, in a long-term, double-blind, randomized controlled trial in which healthy elderly subjects were supplemented with the fish fatty acids EPA and DHA or a placebo for 26 weeks (**Chapter 4**) and in a single-blind, cross-over postprandial challenge study, in which healthy young men consumed shakes containing different fatty acids (**Chapter 5**). In **chapter 6** the results described in the previous chapters are discussed and evaluated.

Chapter 2

Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid β-oxidation: functional role of peroxisome proliferator-activated receptor α in human peripheral blood mononuclear cell

Mark Bouwens, Lydia A. Afman and Michael Müller

Published in: The American Journal of Clinical Nutrition 2007; 86(5):1515–23
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Abstract

Background

Peripheral blood mononuclear cells (PBMC) are the only readily available cells in healthy humans. Various studies showed disease-characteristic gene expression patterns in PBMC. However, little is known of nutritional effects on PBMC gene expression patterns. Fatty acids (FAs) are nutrients that regulate gene expression by e.g. activating the nuclear receptor peroxisome proliferators-activated receptor alpha (PPARα). PBMC express PPARα, making these cells interesting to study FA-dependent gene expression.

Objective

The aim of this study is to elucidate whether PBMC gene expression profiles also reflect nutrition-related metabolic changes. Furthermore, we focused on the specific role of PPARα in regulation of PBMC gene expression during fasting, when plasma free FAs are elevated.

Design

Four healthy male volunteers fasted for 48 hours. PBMC RNA was hybridized on Affymetrix whole genome microarrays. To elucidate the role of PPARα, PBMC of 9 donors were incubated with the specific PPARα ligand Wy14,643.

Results

After 24 and 48 hours fasting, 1,200 and 1,386 genes were changed more than 1.4 fold, respectively. Many of these genes were involved in FA β-oxidation and are known PPARα target genes. Incubation of PBMC with Wy14,643 resulted in up-regulation of genes that were also up-regulated during fasting.

Conclusions

We conclude that PBMC gene expression profiles reflect nutrition-related metabolic changes such as fasting, and that part of the fasting-induced changes are likely regulated by PPARα.

Introduction

White blood cells travel through the entire body and respond to various internal and external signals. A subset of white blood cells known as peripheral blood mononuclear cells (PBMC) receives increased interest for diagnostic purposes since the development of sophisticated transcriptomics techniques such as whole genome microarray analysis. The advantage of PBMC above other cells is that blood is the most readily accessible cellular material in humans and PBMC can be isolated from whole blood relatively easily. Various studies have shown that PBMC can display gene expression patterns characteristic for certain diseases, such as acute myeloid leukemia, atherosclerosis and auto-immune diseases[7-9, 15, 33]. From physiological studies we know that nutritional effects on parameters of human physiology are generally smaller than pathophysiological changes. Similarly, nutritional-induced changes in PBMC gene expression are expected to be smaller than the described effects observed in various diseases. Characterization of nutrient-specific effects on cellular metabolism and homeostatic control could in the end lead to evidence-based dietary advice in order to prevent disease or intervene in a very early pre-disease state. The comprehensive understanding of the mechanisms involved in nutritional effects on gene expression is of considerable importance in this respect. This is especially the case in PBMC, since obtaining alternative cellular material, through invasive tissue biopsies, is not always feasible or even possible in healthy volunteers, because of ethical reasons. Therefore, the ability to detect diet-induced changes in gene expression profiles of relatively easily obtainable PBMC could not only be highly valuable for the identification of nutrient-related molecular mechanism, but will also be of pivot importance to study the development of diet-related chronic disorders. It is known that several nutrients are able to activate gene expression by binding to transcription factors[1]. This is quite well understood for fatty acids (FA)[34], which mediate their modulatory effects on changes of expression levels of specific genes by a group of nuclear receptors, the peroxisome proliferators-activated receptors (PPAR). These ligand-activated transcription factors (PPARα, PPARβ/δ, PPARγ) bind FA, in particular if they are unsaturated, which results in transcriptional activation. Activation of PPARα occurs e.g. when plasma free fatty acid (FFA) concentrations are increased in situations such as fasting and heavy exercise, postprandially and in conditions such as diabetes type II and visceral obesity. Fasting results in the most pronounced elevation in plasma FFA, due to breakdown of triacylglycer-

ols stored in the adipose tissue[35]. Studies in liver from fasted mice showed that PPARα transcriptionally activates genes involved in processes essential for utilization of increased FA concentrations for energy, such as β-oxidation[36]. Since previous studies showed that PPARα is expressed in human PBMC[31, 32, 37], and FA are abundantly present in blood plasma of fasting humans, these nutrients are excellent candidates to study the feasibility of detecting and analyzing nutrients-induced changes in PBMC gene expression profiles. In order to assess this, we conducted a study in which four healthy men fasted for 48 hours, monitored changes in PBMC gene expression during fasting by whole genome microarray analysis, and examined the role of PPARα in this respect.

Subjects, Materials and Methods

Recruitment of subjects

Four healthy male Caucasian volunteers, between 19 and 22 years of age were recruited from the Wageningen student population. Physiologically measured exclusion criteria were a body mass index (BMI) < 19 or > 25 kg/m2, urine protein or glucose concentrations outside normal ranges or fasting blood glucose outside the normal range. Other exclusion criteria were: tobacco smoking, taking regular prescribed medication, received inoculations within 2 months of starting the study or planned to during the study, donated or intended to donate blood within 8 weeks of the first and last study samples, diagnosed with any long-term medical condition (eg., diabetes, hemophilia, cardiovascular disease, anemia, gastrointestinal disease), experiencing symptoms of allergy or vegetarian. Subjects were informed about the design and purpose of the study and provided full informed written consent.

Study design of the intervention study

At a screening visit, urine and fasting venous blood samples were taken to determine glucose concentrations in both urine and blood, and protein concentrations in urine. Four healthy male subjects who fulfilled selection criteria were included in the study. Volunteers received an identical meal at 5 pm, before the start of a 48 hour fasting period. During the fasting period, the subjects were not allowed to eat or drink anything except water, which they could consume ad libitum. After 48 hours, the volunteers received a light meal. After 0, 24 and 48 hours of fasting, 45 ml of blood was drawn into EDTA tubes from the antecubital vein in

each volunteer's forearm. Volunteers were asked to keep record of their physical condition and after 15 hours of fasting they remained under constant supervision. The total study was repeated two months later with the same subjects. The study protocol was approved by the Medical Ethical Committee of Wageningen University.

Blood glucose and plasma free fatty acids

Blood glucose concentrations were determined with Accu-Chek Compact blood glucose meters (Roche Applied Science). Immediately after blood drawing, blood was centrifuged and plasma was stored at -80˚ C. Plasma free fatty acids were measured by gas–liquid chromatography (GLC)[38, 39]. Briefly, plasma was incubated with a modified Dole reagent (2-propanol/heptane/2 M orthophosphoric acid, 40:10:1) for 10 min a room temperature. Heptane was added and the tubes were mixed (5 minutes) and centrifuged at 3000 rpm for 5 minutes. The upper heptane layer was transferred to another tube and evaporated under nitrogen. The residue was solved in chloroform and FFA were separated from the triacylglycerols and phospholipids on amino propyl columns. The FFA were hydrolyzed in 0.5 N methanolic sodium-hydroxide and methylated with borontrifluoride in methanol. The methyl esters were separated on a glass capillary column and measured with a flame ionization detector. Identification of individual methyl esters was performed by comparisons with authentic standard mixtures. Heptadecanoic acid (C17:0) was used as an internal standard for quantification of individual free fatty acids.

PBMC isolation for the intervention study

Immediately after blood collection, PBMC were isolated out of 36 ml blood, from all time points and subjects for both study periods, using Optiprep (Axis-shield, Oslo, Norway) according to the manufacturer's manual.

PBMC isolation for cell culture

Buffy coats from 9 blood donors were acquired through the blood bank (Sanquin, Nijmegen, the Netherlands). Blood donors were healthy Caucasian men, aged between 18 and 40 yr, which gave informed consent. PBMC were isolated directly after arrival of the buffy coat (at most 8 hours after donation) by Ficol-paque Plus density gradient centrifugation (Amersham Biosciences, Roosendaal, the Nether-

lands). Cells were washed twice in RPMI1640 medium and isolated cells were counted and tested for viability by trypan blue exclusion prior to culture.

PBMC cell culture

PBMC were cultured using RPMI1640 medium with 2 mmol/L L-glutamine, 10% fetal bovine serum and antibiotics (penicillin and streptomycin) in the presence of 5% CO2 at 37°C. Cell culture medium containing glutamine and serum were purchased from Gibco BRL (Grand Island, NY). PBMC were incubated at 1.0 x 106 cells per ml with 50 µmol/L Wy14,643, or 0.1% DMSO (vehicle), for 12 hours. Following incubation, the cells were collected, washed twice in phosphate-buffered saline, and counted and tested for viability by trypan blue exclusion.

Flow cytrometric analysis of PBMC vitality

Viability of the cells after incubation with 50 µmol/L Wy14,643 was determined by staining with annexin/ propidium iodide (PI) followed by flow cytometry analysis with a Coulter Epics XL.MCL flow cytometer (Beckman Coulter).

Total RNA isolation

PBMC RNA was isolated from all PBMC samples using Qiagen RNeasy Micro kit (Qiagen, Venlo, the Netherlands). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (Nanodrop technologies, Wilmington, Delaware USA) and integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, UK).

Microarray processing

From the twelve PBMC RNA samples from the first study, eleven samples were used for microarray processing. One sample from the first study was excluded due to low RNA quality. One microgram of total RNA per sample was labelled using a one-cycle cDNA labelling kit (Affymetrix Inc, Santa Clara, CA) and hybridized to Affymetrix Human whole genome U133 plus 2.0 arrays (Affymetrix). Sample labeling, hybridization to chips and image scanning was performed according to the manufacturer's GeneChip Expression Analysis Technical Manual (Affymetrix).

Microarray data analysis

Quality control was performed and fulfilled the criteria for array hybridization suggested by the Tumor Analysis Best Practices Working Group[40]. Expression values were calculated using the Robust Multichip Average (RMA) method. RMA signal value estimates are based on a robust average of background corrected perfect match intensities and normalization was done using quantile normalization[41, 42]. Probe sets were filtered using the Inter Quartile Range (IQR)[43], using a cut off value of 0.3. Only probe sets with normalized signals higher then 20 on at least two arrays were defined as expressed and selected for further analysis. Individual probe sets were defined as 'changed' when comparison of the normalized signal intensities showed a p-value lower then 0.05 in a two-tailed paired t test. For pathway analysis, only probe sets with an average fold change of >1.4 or <-1.4 between 0 and 24 hours, or 0 and 48 hours were selected. Conversion of number of probe sets to amount of genes was performed by using the DAVID Gene ID conversion tool (DAVID Bioinformatic Resources 2006, http://niaid.abcc.ncifcrf.gov/conversion.jsp). Pathway analysis was performed using GenMAPP 2.0[44] (http://www.genmapp.org/). The MAPPFinder software was used to rank the GenMAPP local pathways by z-score during fasting. Z-scores in this software program are calculated by subtracting the expected number of genes meeting the criterion from the observed number, divided by the standard deviation of the observed number of genes.

cDNA synthesis and Quantitative Real Time PCR

RNA was reverse transcribed using the iScript cDNA synthesis kit (Biorad). Standard quantitative real time polymerase chain reaction (Q-PCR) was performed using Platinum Taq DNA polymerase and SYBR Green on an iCycler PCR machine (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) and duplicated at least twice. Primer sequences used in the PCRs were chosen based on the sequences available in PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html). Q-PCR data were normalized by measuring cycle threshold (Ct) ratios between candidate genes and a housekeeping gene, human acidic ribosomal phosphoprotein PO (HuPO), which was shown to be consistent within PBMC[45].

Statistical methods

A two-tailed paired t test was used to determine significant differences in plasma

metabolite concentrations and Q-PCR gene expression values between baseline and 24 hours of fasting, and between baseline and 48 hours of fasting. We applied Bonferroni corrections as an adjustment for multiple testing and defined statistical significance at $p<0.025$. For the *ex vivo* experiments statistical significance was accepted at $p<0.05$. All calculations were performed by using the SPSS-software package 12.0.1 (Chicago, Illinois).

Results

Volunteer characteristics

All four volunteers completed both 48 hour fasting periods without any problems. The characteristics of the subject population are presented in **Table 1**, in which the glucose concentrations are measured during the screening after an overnight fast. Fasting during the study resulted in a decrease in glucose concentrations, from an average of 4.2 mmol/L directly after the meal, to an average of 3.6 mmol/L after 48 hours (data not shown). Changes in individual free fatty acids are shown in **Figure 1**. All plasma free fatty acid concentrations were strongly elevated after 24 hours fasting and remained elevated after 48 hours fasting. The mean increases after 48 hours fasting in mono-unsaturated fatty acids (MUFA), saturated fatty acids (SFA) and poly-unsaturated fatty acids (PUFA) were 10, seven and six times the basal concentration, respectively (**Figure 1A**). Within the PUFA the n-3 and n-6 fatty acids increased four and six times within 48 hours fasting (**Figure 1B**).

Table 1 Subject characteristics of the men participating in the study.

	Mean (n=4)	Range
Age (y)	20.6	19 – 22
Height (m)	1.84	1.80 – 1.87
Weight (kg)	72	68 – 79
BMI (kg/m2)	21.3	20.4 – 22.6
Fasting glucose (mmol/L) *	5.2	4.9 – 5.7

* Glucose values were determined after an overnight fast during the screening visit

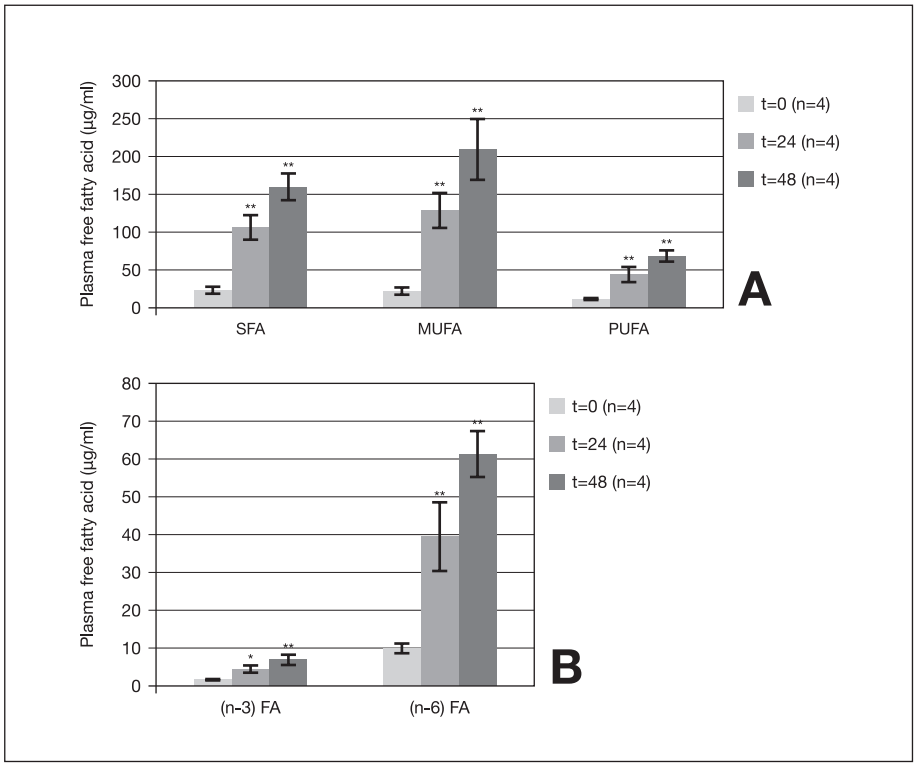

Figure 1 Mean plasma free fatty acid concentrations during fasting. A. SFA, Saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acid. B. n-3 FA, omega-3 fatty acids; n-6 FA, omega-6 fatty acids. Asterisks indicates significant change compared to t=0 (* $p < 0.025$; ** $p < 0.01$) as determined with a paired t-test. Bars represent mean ± SD (n=4).

Differential gene expression on microarray

Fasting for 24 and 48 hours resulted in a differential expression of 3,582 out of a total of 54,675 tested probe sets in PBMC (**Figure 2**). These changed probe sets encoded for 3,030 genes. After selection of probe sets with a signal higher then 20 in at least 2 arrays and a fold change higher than 1.4 or lower than -1.4, 1,200 genes were changed after 24 hours and 1,386 genes after 48 hours. Most of these genes showed an up-regulation in expression, i.e. 74% after 24 hours and 78% after 48 hours. Of the genes meeting the selection criteria more than 74% were changed at both time points.

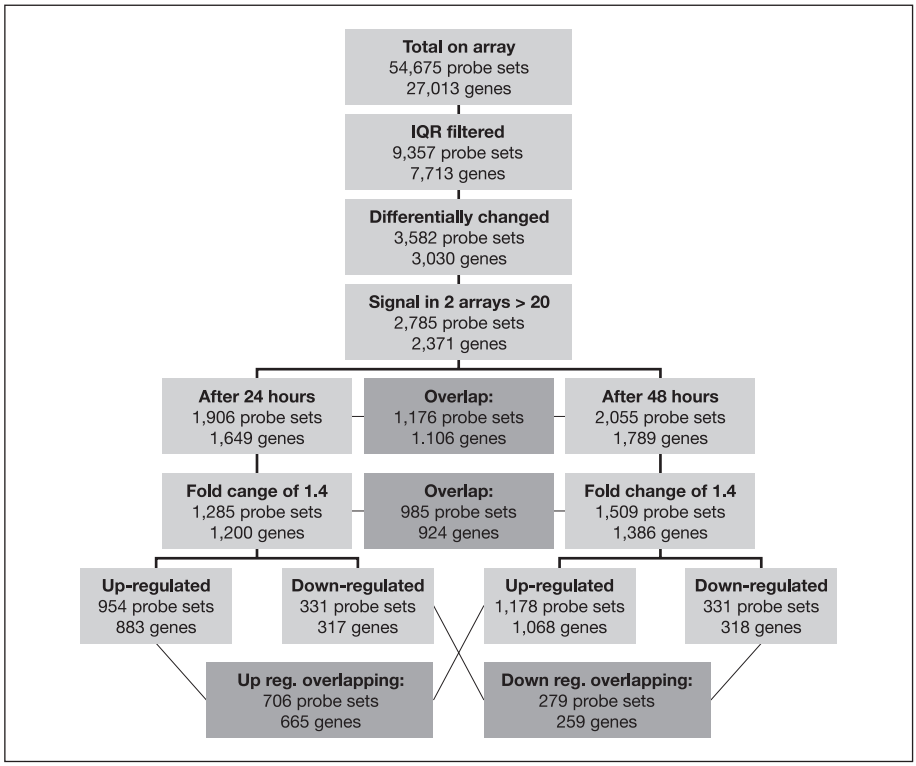

Figure 2 Stepwise probe set selection in microarray analysis.

Pathway analysis

Subsequent pathway analysis of the microarray data revealed that several genes that exhibited increased expression upon fasting were involved in fatty acid metabolism and, more specifically, in fatty acid β-oxidation (**Figure 3**). Furthermore, pyruvate metabolism and most of the RNA/DNA metabolism pathways were up-regulated upon fasting in PBMC. A decreased expression of genes involve in the TCA cycle was observed. Most pathways were already changed after 24 hours, which sustained until 48 hours. Exceptions were regulation of viral genome replication and B-cell differentiation, which only showed up-regulation after 48 hours and heme biosynthesis, which only showed down-regulated at 48 hour. Another exception was negative regulation of transcription, which showed up-regulated and down-regulation of genes in the same "pathway", leading to both an increase and decrease at 24 hours.

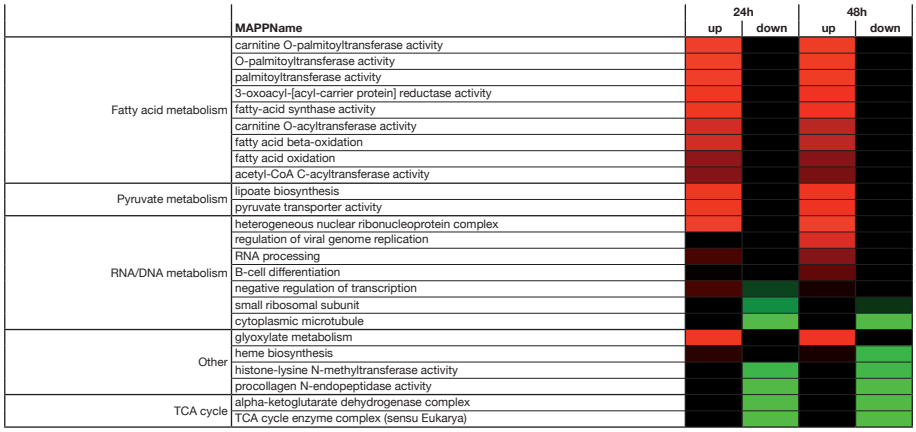

Figure 3 Heat map of changed pathways after 24 hours and 48 hours of fasting in peripheral blood mononuclear cells ranked by Z-scores (GeneMAPP 2.0). Only pathways with z-scores higher than 1 at either 24 or 48 hours were included. Z-scores for down-regulated pathways were inverted. Red indicates up-regulated, green indicates down-regulated.

Specific gene expression

Of all the genes changed after 24 and 48 hours of fasting (**additional file**), pyruvate dehydrogenase kinase, isoform 4 (PDK4) showed the largest changes in gene expression with increases of 7.2 and 7.4 after 24 and 48 hours fasting, respectively. This gene is known to be regulated by PPARα. **Table 2** shows a list of the genes which expression we found to be changed after fasting and that are known PPARα target genes from mice studies. Interestingly, expression of all these PPARα target genes was up-regulated, either at 24 hour, 48 hours or at both time points. Many of these genes are involved in fatty acid β-oxidation, such as carnitine palmitoyltransferase 1 (CPT1), acetyl-Coenzyme A acyltransferase 2 (ACAA2), solute carrier family 25 (carnitine/acylcarnitine translocase), member 20 (SLC25A20), acyl-Coenzyme A dehydrogenase, very long chain (ACADVL) and acyl-Coenzyme A thioesterase 2 (ACOT2).

Table 2 Known PPARα target gene expression in peripheral blood mononuclear cells on the microarrays.

Affy ID	Genename	Description	Mean signal	Mean FC 24vs0[1,2]	Mean FC 48vs0[1,2]
225207_at	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	>50	7.19	7.40
205960_at	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	>50	7.10	6.81
203633_at	CPT1A	carnitine palmitoyltransferase 1A (liver)	>50	2.05	2.08
210688_s_at	CPT1A	carnitine palmitoyltransferase 1A (liver)	20-50	1.75	1.94
210069_at	CPT1B	carnitine palmitoyltransferase 1B (muscle)	>50	1.79	1.73
210070_s_at	CPT1B	carnitine palmitoyltransferase 1B (muscle)	20-50	NC	1.40
203658_at	SLC25A20	solute carrier family 25 (carnitine/ acylcarnitine translocase), member 20	>50	1.93	1.87
209122_at	ADFP	adipose differentiation-related protein	>50	1.93	1.85
202003_s_at	ACAA2	acetyl-Coenzyme A acyltransferase 2	>50	1.61	1.61
200710_at	ACADVL	acyl-Coenzyme A dehydrogenase, very long chain	>50	1.47	1.46
213524_s_at	G0S2	G0/G1switch 2	>50	4.80	NC
202982_s_at	ACOT2	acyl-CoA thioesterase 2	20-50	NC	1.53

[1] All fold changes (FC) are significant ($p<0.05$).
[2] All genes were up-regulated. NC indicates not changed significantly.

Confirmation of gene expression data

To confirm the data found with microarray analysis and to observe whether the changes were consistent, quantitative real time PCR (Q-PCR) was performed on RNA from the first and the second study (Figure 4). Genes chosen for confirmation were PDK4 and several genes involved in fatty acid β-oxidation. For all genes the mode of change observed with microarray analysis could be confirmed by Q-PCR, although changes were smaller. Q-PCR changes after 48 hours of fasting, but not after 24 hours, were significant for PDK4 and SLC25a20. In the second study, all genes were significantly changed at 48 hours, except for CPT1, which showed no significant change. Changes at 24 hours in the second study were increased significantly for PDK4 and SLC25A20 (**Figure 4**).

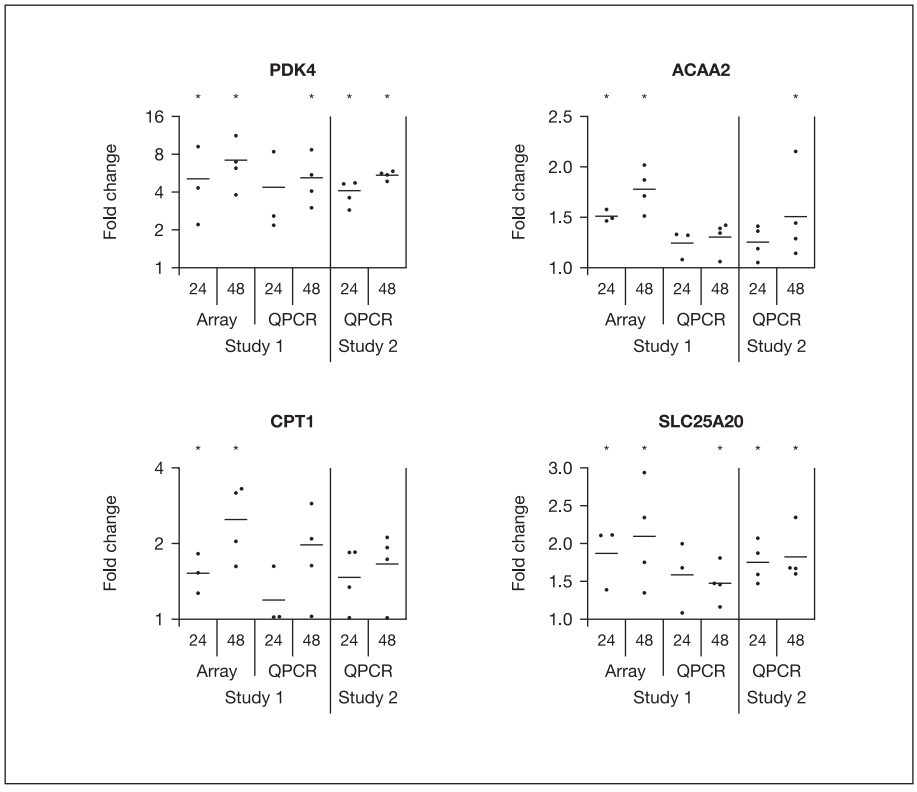

Figure 4 Microarray and real-time gene expression changes of four PPARα target genes after 24 and 48 hours fasting in both intervention studies. Dots represent fold changes of the individual subjects and lines represent mean values. P-values were determined with a paired *t*-test. Asterisk indicates significant change compared to t=0 (p <0.025).

Cell culture experiments

Several of the genes changed upon fasting are known PPARα target genes. To reveal whether those genes can be activated via PPARα in PBMC, freshly isolated PBMC from 9 healthy male donors were used in cell culture experiments. Incubation of these PBMC with 50 µmol/L of the highly specific PPARα ligand Wy14,643 revealed an increase in expression of PDK4, CPT1, ACAA2 and SLC25A20, compared to the control cells, which were incubated with vehicle (**Figure 5**). PPARα expression itself was not changed upon incubation with Wy14,643. PBMC showed no loss of viability after incubation with 50 µmol/L of Wy14,643 for 12 hours (data not shown).

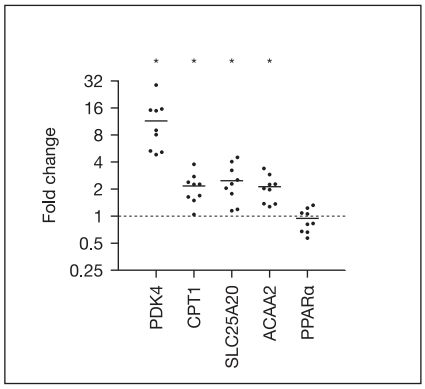

Figure 5 Changes in gene expression of four PPARα target genes and PPARα in PBMC of nine donors after incubation with 50µmol/L of Wy14,643 for 12 hours. Dots represent different donors, lines represent mean values. * significantly changed compared to vehicle (p <0.01) as determined with a paired t-test.

Discussion

Here we demonstrate that elevated concentrations of plasma free fatty acid during fasting have pronounced effect on PBMC gene expression, in particular on expression of PPARα target genes. Correspondingly, pathway analysis showed a strong increase in genes involved in fatty acid metabolism, and, more specific, of the fatty acid β-oxidation in PBMC. The functionality of PPARα in PBMC was demonstrated in an *ex-vivo* study in which we showed that PPARα is responsible for an increased transcription of genes involved in fatty acid β-oxidation when these cells are incubated with the highly specific PPARα ligand Wy14,643.

So far, mainly disease-related PBMC genomic fingerprints have been revealed with transcriptomics techniques[7-9, 15, 33]. We observed that even during a metabolic condition such as fasting, when fatty acids are elevated, microarray analyzes can reveal many differentially expressed genes in PBMC. These gene expression changes are not only observed after 48 hours, but are already present after 24 hours of fasting. The clear overlap in genes changed at both time points is also reflected in a similar change in pathways after 24 hours and 48 hours of fasting. Interestingly, the number of genes changed in PBMC is quite large, especially if the low number of four subjects included in the study is taken into account. To assure that the gene expression changes were truly due to fasting, the whole study was repeated two months later with the same persons. In this second study, we observed that several of the genes, which expression was changed in the first study, were also changed, demonstrating the consistency of our microarray findings. In addition, the high number of genes changed and the consistency of the findings indicate that fasting has a profound effect on gene expression in PBMC. These promising results implicate that PBMC may also be used to monitor more subtle effects associated with dietary intervention, providing that sufficient numbers of subjects are included.

While a large number of genes were changed after fasting, many of the genes changed play a role in metabolic pathways normally regulated during fasting in the liver, such as an increase in fatty acid β-oxidation and a decrease in the TCA-cycle[35]. During fasting, when free fatty acids are used as the main fuel source, fatty acid β-oxidation will be up-regulated to provide acetyl coenzyme A (CoA), subsequently used in the TCA-cycle. Our study showed that genes involved in fatty acid β-oxidation were also up-regulated in PBMC after fasting. Interestingly, as known from mice studies, the expression of several of these genes is regulated

by the fatty acid-activated nuclear receptor PPARα. In these mice studies, fasting has been shown to activate PPARα in liver, resulting in up-regulation of processes such as fatty acid β-oxidation[36]. Moreover, the gene with the most profound up-regulation, pyruvate dehydrogenase kinase (PDK)4, is also known to be regulated by PPARα during fasting in rat skeleton muscle and heart[46, 47]. PDK4 executes its role during fasting by inhibiting the pyruvate dehydrogenase complex (PDC), thereby decreasing conversion of pyruvate to acetyl CoA leading to conservation of glucose. PDK4 is expressed at high levels in various tissues, predominantly in heart and skeletal muscle, but also liver and kidney[48]. The role of human PDK4 during fasting has mainly been studied in skeletal muscle, in which, similar to our study, PDK4 expression was increased[49-51]. The functionality of PPARα, with respect to its capability to increase the expression of genes involved in fatty acid β-oxidation and PDK4 in PBMC, was proven by the *ex vivo* incubation with the specific PPARα ligand Wy14,643. We showed that these PPARα target genes were up-regulated in PBMC from 9 different donors after incubation with the ligand. This indicates that PPARα is functional in PBMC and likely mediates the fatty acid dependent gene expression in these cells. It has previously been reported that PPARα-controlled genes in mice T-cells could only be up-regulated by ligand-induced activation of PPARα when histone deacetylase inhibitors are utilized[52]. Interestingly, we found an up-regulation of PPARα target genes in freshly isolated human PBMC, consisting of roughly 60% T-cells, without addition of histone deacetylase inhibitors. In addition, it has also been reported that activation of PPARα during fasting or with the PPARα ligand Wy14643 results in an up-regulation in expression of PPARα itself in liver tissue of mice studies and from *in vitro* studies with human primary hepatocytes[36, 53]. We however did not observe such auto-regulation of PPARα in human PBMC, neither during fasting nor after incubation of the cells with Wy14,643. Both studies showed no change in gene expression of PPARα. We speculate that this auto-regulation is less pronounced in or not present in PBMC, which may explain the discrepancies between the studies performed in either mouse or other human tissues.

We found in our study a sizable variation in both basal expression of the genes, as in gene expression changes, between the subjects. This not only points towards a large variation between persons, but also to a large variety in the gene expression response towards a metabolic change. From microarray studies it is reported that not only variation in PBMC basal gene expression between human subjects

can be very large, but also that there is a sizable variation in proportions of PBMC subpopulations between individuals, which might explain the large variation in individual response[17-19]. However, all studies report that within one individual, variety is relatively small, both in PBMC gene expression and subpopulations, and has little effect on PBMC gene transcription profiles. Therefore, dietary intervention studies with multiple measurements within the same individual are necessary for the detection of gene expression changes in PBMC.

Concisely, our study shows that during fasting, blood free fatty acids are elevated, activating PPARα in the blood cells i.e. the PBMC, thereby inhibiting the conversion of pyruvate to acetyl CoA to conserve glucose, by up-regulating PDK4 and at the same time increasing fatty acid β-oxidation to provide energy by metabolizing the released free fatty acids. This elevated fatty acid β-oxidation increases the lipid handling capacity of PBMC. From all fatty acids, polyunsaturated fatty acids have been shown to activate PPARα most profoundly[25, 26]. We therefore hypothesise that these fatty acids will have the largest effect on PPARα-dependent gene transcription and subsequently increase lipid handling capacity in PBMC to the highest extend. Moreover, it has been found that activation of T-cells after pre-incubation with a PPARα ligand reduces expression of pro-inflammatory cytokines[31]. Both, the cellular lipid handling and the balanced inflammatory response capacity, are of pivot importance in the development of diet-related chronic diseases such as metabolic syndrome, diabetes type II and atherosclerosis. Illustrative for this is that PPARα-specific agonists strongly inhibited atherosclerosis in mice studies[54, 55]. In addition, several clinical trials have shown that synthetic agonists of PPARα have beneficial, anti-atherogenic effects in humans[56]. We hypothesize that replacing dietary saturated fatty acids by dietary (poly)unsaturated fatty acid will activate PPARα, leading to an increased fat handling capacity and decreased pro-inflammatory response in PBMC, ultimately resulting in a decreased cell activation and consequent prevention of the onset of diet-related disorders such as atherosclerosis and metabolic syndrome. These results not only imply that PBMC can be used for further characterization of human PPARα, *in vivo* and *ex vivo*, but also that PBMC are good candidate cells to study other molecular mechanism of nutrients. In summary, we have shown that physiologically relevant metabolic conditions such as fasting are reflected by PBMC gene expression profiles. Therefore, these profiles show great potential to be used in the detection of even more subtle changes in

gene expression as expected in nutritional intervention studies. Additionally, our intervention study showed that during fasting, when blood free fatty acids are increased, the fat handling capability of PBMC was augmented by an increase in fatty acid β-oxidation. Finally, our *ex vivo* study demonstrated a functional role of PPARα in human PBMC, suggesting that this nuclear factor regulates the lipid handling capacity of these cells, which probably can be regulated by the type of dietary fat intake.

Authors contributions

MB collected and analyzed the data and wrote the manuscript. LAA an MM designed the studies and participated in critical revising of the manuscript. None of the authors had a personal or financial conflict of interest.

Additional file can be found online:

www.ajcn.org/cgi/data/86/5/1515/DC1/1

Genes changed over 1.4 fold after 24 hours and/or 48 hours of fasting. FC, fold change

Chapter 3

Activation of peroxisome proliferator-activated receptor α in human peripheral blood mononuclear cells reveals an individual gene expression profile response

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Published in: BMC Genomics 2008, 9:262.
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Abstract

Background

Peripheral blood mononuclear cells (PBMCs) are relatively easily obtainable cells in humans. Gene expression profiles of PBMCs have been shown to reflect the pathological and physiological state of a person. Recently, we showed that the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) has a functional role in human PBMCs during fasting. However, the extent of the role of PPARα in human PBMCs remains unclear. In this study, we therefore performed gene expression profiling of PBMCs incubated with the specific PPARα ligand WY14,643.

Results

Incubation of PBMCs with WY14,643 for 12 hours resulted in a differential expression of 1,373 of the 13,080 genes expressed in the PBMCs. Gene expression profiles showed a clear individual response to PPARα activation between six healthy human blood donors. Pathway analysis showed that genes in fatty acid metabolism, primarily in β-oxidation were up-regulated upon activation of PPARα with WY14,643, and genes in several amino acid metabolism pathways were down-regulated.

Conclusions

This study shows that PPARα in human PBMCs regulates fatty acid and amino acid metabolism. In addition, PBMC gene expression profiles show individual responses to WY14,643 activation. We showed that PBMCs are a suitable model to study changes in PPARα activation in healthy humans.

Background

The function of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) has been studied extensively from the time of its discovery in the early 1990s[57]. PPARα is a ligand activated nuclear receptor, which is known to be activated by free fatty acids and their derivatives[58, 59]. Besides fatty acids, several synthetic compounds are available that specifically activate PPARα, including hypolipidemic drugs, such as fibrates and pirinixic acid (WY14,643)[60]. Synthetic PPARα agonists mimic effects of dietary unsaturated fatty acids on hepatic gene expression in terms of regulation of target genes and molecular mechanism[61]. Activation of PPARα with WY14,643 in mice showed that the main function of PPARα in liver is the regulation of lipid metabolism, and more specifically fatty acid β-oxidation[62]. PPARα was also found to be involved in regulation of amino acid metabolism[63] and inflammation[64, 65]. In humans, the function of PPARα is examined less thoroughly, because functional studies are more complicated. There is no human genetic disease linked to a dysfunctional *PPARα* gene and tissue sampling is often not feasible in healthy volunteers. Blood is one of the few tissues which is readily available in healthy humans. Peripheral blood mononuclear cells (PBMCs) are relatively easily obtainable by isolation from blood. These cells consist of lymphocytes and monocytes/macrophages and it is known that PPARα is expressed in these cells[31, 32]. The use of PBMCs has proven to be highly robust in distinguishing a disease state from healthy state, by studying gene expression profiles of these cells[7, 66]. Recently, we showed that PBMC gene expression profiles of healthy volunteers can also reflect changes between 24 and 48 hours fasting, when plasma fatty acid concentrations are elevated. In addition, we showed that PPARα seems to have a functional role in human PBMC during fasting as several of the genes changed upon fasting were also changed upon incubation of PBMC with the specific PPARα agonist WY14,643[67]. However, the extent of the role of PPARα in human PBMCs remains unclear. Therefore, we tried to elucidate the function of PPARα in human PBMCs by whole genome microarray analysis of the PBMCs incubated with the specific PPARα ligand WY14,643. Furthermore, to examine the complete role of PPARα within PBMCs during fasting, we compared microarray analysis of PBMCs activated with WY14,643, with microarray analysis of PBMCs during 24 hours of fasting.

Methods

PBMC incubation

PBMCs from six healthy Caucasian male blood donors, aged between 30 and 48 yr, were isolated directly after arrival of the buffy coat (max. 8 hours after donation) by Ficol-paque Plus density gradient centrifugation (Amersham Biosciences, Roosendaal, the Netherlands). PBMCs were incubated in RPMI1640 medium with 2 mmol/L L-glutamine, 10% fetal bovine serum and antibiotics (penicillin and streptomycin) in the presence of 5% CO_2 at 37°C. at 1.0 x 10^6 cells per ml with either WY14,643 (50 μM) or vehicle (DMSO, 0.1%) for 12 hours. All donors gave full written informed consent.

Pre- vs. postprandial incubation

PBMCs of four healthy volunteers, aged between 28 and 34, were isolated after a meal and after an overnight fast. PBMCs were incubated at 1.0 x 10^6 cells per ml with either WY14,643 (50 μM) or vehicle (DMSO, 0.1%) for 12 hours. All volunteers gave full written informed consent.

Statistical methods

A 2-tailed paired *t* test was used to determine significant differences in Q-PCR gene expression values between the postprandial and the fasted state. Statistical significance was accepted at *p* 0.05. All calculations were performed with the use of the SPSS (version 12.0.1; SPSS, Chicago, IL).

Microarray processing

For 6 donors of the incubation experiments, total RNA from PBMCs was labeled using a one-cycle cDNA labeling kit (Affymetrix Inc, Santa Clara, CA) and hybridized to Affymetrix Human whole genome U133 plus 2.0 arrays (Affymetrix). Sample labeling, hybridization to chips and image scanning was performed according to the manufacturer's GeneChip Expression Analysis Technical Manual (Affymetrix).

Intervention study

For comparison of microarray data of the abovementioned incubation study, with microarray data of PBMC of fasted volunteers, we used the earlier described microarray data of a 48 hours fasting study[67]. Briefly, four healthy male Caucasian

volunteers, between 19 and 22 year of age were fasted for 48 hours. PBMCs were isolated out of blood taken at baseline, after 24 hours and after 48 hours of fasting. All volunteers gave full written informed consent and the study protocol was approved by the medical ethics committee of Wageningen University.

Microarray analysis

Microarrays were analyzed using the reorganized oligonucleotide probes as described by Dai *et al* (2005)[68]. Dai *et al.* combined all individual probes for a gene, enabling the possibility to detect the overall transcription activity of a gene, based on the latest genome and transcriptome information, instead of the Affymetrix probe set annotation. Application of this annotation procedure on the previously published data from the 48 hours fasting study[67] resulted in a difference in number of genes expressed and changed as compared to the previously used annotation method as this analysis was performed on probe set level.

Expression values were calculated using the Robust Multichip Average (RMA) method. RMA signal value estimates are based on a robust average of background corrected perfect match intensities and normalization was performed using quantile normalization[41, 42]. Only genes with normalized signals higher then 20 on at least two out of twelve arrays were defined as expressed and selected for further analysis. Genes were defined as 'changed' when comparison of the normalized signal intensities showed a p-value lower then 0.05 in a two-tailed paired t test with Bayesian correction (Limma)[69]. Pathway analysis was performed using Ingenuity Pathway Analysis 5.5 (Ingenuity Systems). Array data have been submitted to the Gene Expression Omnibus, accession number GSE11289.

PPRE incidence

To indicated which of the genes changed upon activation of PPARα had a predicted or reported peroxisome proliferator response element (PPRE), we used information from Lemay *et al.*[70]. This paper recently reported predicted PPRE on a genome wide scale, using computational genomics and also summarized known PPRE. Using Genomatix software[71], network analysis was performed on 1,373 genes in BiblioSphere, from which transcription factors were selected that were directly linked to more than 10 genes from our list of changed genes. Subsequent transcription factor binding site analysis identified transcription factor binding

sites in the promoters of our genes of interest that were cocited at least once in an abstract with these transcription factors._Heat maps were created by using Spotfire software.

cDNA synthesis and quantitative real-time PCR

RNA was reverse transcribed with the use of the cDNA synthesis kit (Promega, Leiden, the Netherlands). Standard quantitative real-time polymerase chain reaction (Q-PCR) was performed with the use of Platinum Taq DNA polymerase and SYBR Green on an iCycler PCR machine (Bio-Rad Laboratories BV) and duplicated at least twice. Primer sequences used in the PCRs were chosen based on the sequences available in PRIMERBANK[72]. Q-PCR data were normalized by measuring cycle threshold ratios between candidate genes and a housekeeping gene, human acidic ribosomal phosphoprotein PO, which was shown to be consistent within PBMCs[45].

Results

PPARα regulation in PBMCs after incubation with WY14,643

Incubation of PBMC with the specific PPARα ligand WY14,643 for 12 hours resulted in a differential expression of 1,373 of the 13,080 genes expressed in the PBMCs, indicating a PPARα-dependent regulation of 10.5% of the genes expressed in PBMC (**Figure 1**). More than half of these genes (56%) were up-regulated. Pathway analysis of the genes changed upon activation of PPARα with WY14,643, showed a marked increase in fatty acid metabolism, primarily in β-oxidation, and a decrease in several amino acid metabolism pathways (data not shown).

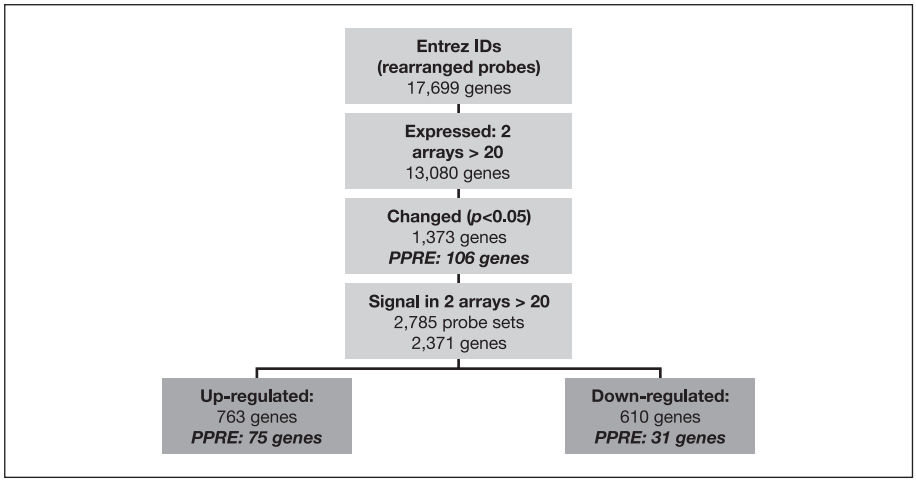

Figure 1 Flow chart of the followed gene selection procedure after microarray analysis of WY14,643 incubated PBMCs from 6 donors. PPRE; number of genes containing a peroxisome proliferator response element according to Lemay *et al.*

A peroxisome proliferator response elements (PPREs) was ascribed to 106 out of the 1,373 genes changed, using the study of Lemay *et al*[70]. Of these genes, 75 were up-regulated and 31 were down-regulated (**Figure 2**). Figure 2 shows the responses to activation of PPARα for each person by illustrating the changes in gene expression of these 106 genes per individual. For several genes a clear variation in response upon PPARα activation between individuals is present. Donor B and, especially, donor E show an obvious distinction from the other donors. However, no difference could be found in the expression of *PPARα* between the donors at basal level, and also after incubation with WY14,643 no change in expression of *PPARα* was observed (data not shown).

Figure 2 (right page) Heatmap of the signal log ratio of genes changed upon incubation with WY14,643 that contained a predicted or reported PPRE. Red indicates up-regulation compared to the vehicle incubated PBMCs and green indicates down-regulation. SLR, signal log ratio; PPRE, peroxisome proliferator response element.

Another reason for the variation could be the difference in concentration of the PPARα ligands, i.e. fatty acids, and other nutritional factors present in the blood during donation. Blood donors are commonly advised to eat before donating blood. To investigate whether the nutritional status influences changes in gene expression, we incubated PBMCs of four volunteers, obtained after a meal and after an overnight fast, with WY14,643. Using QPCR, we determined the changes in PBMC gene expression of genes that showed either a low (*PDK4, SLC25A20, ACAA2*) or a high variation (*CPT1, ABCA1, TLR4, PPARγ*) between donors in the microarray analyzes of the first study (**Figure 3**). No significant differences were observed in gene expression between the fasted and postprandial state.

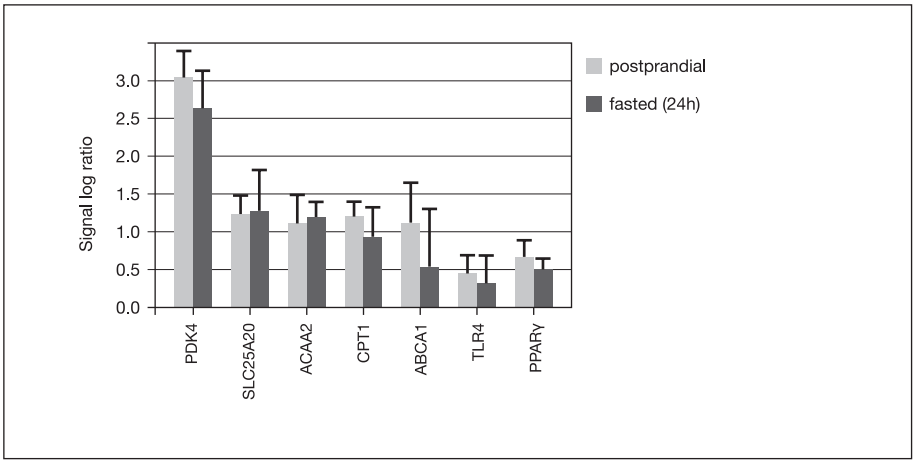

Figure 3 Mean gene expression changes of PBMCs incubated with WY14,643, isolated postprandial or after an overnight fast. Error bars indicate standard deviations. *PDK4, Pyruvate dehydrogenase kinase 4; SLC25A20, carnitine-acylcarnitine translocase; ACAA2, acetyl-Coenzyme A acyltransferase 2; CPT1, Carnitine palmitoyltransferase 1; ABCA1, ATP binding cassette transporter 1; TLR4, Toll-like receptor-4; PPARγ, peroxisome proliferator-activated receptor gamma.*

To analyze whether the genes that did not have a PPRE according to Lemay *et al.* might have other transcriptional binding sites, a network analysis and a

Gene Name	Gene Description	Entrez ID	Mean FC	p-value	PPRE	SLR A	SLR B	SLR C	SLR D	SLR E	SLR F
HAMP	Hepcidin precursor (Liver-expressed antimicrobial peptide)	57817	-1.40	0.00076	predicted						
PTX3	Pentraxin-related protein PTX3 precursor	5806	-1.31	0.00780	predicted						
KCTD12	BTB/POZ domain-containing protein KCTD12	115207	-1.33	0.00037	predicted						
ZEB2	Zinc finger E-box-binding homeobox 2	9839	-1.28	0.00794	predicted						
PAK1IP1	p21-activated protein kinase-interacting protein 1 (PAK1-interacting protein 1)	55003	-1.21	0.03585	predicted						
CCL23	Small inducible cytokine A23 precursor	6368	-1.20	0.01667	predicted						
CCL19	Small inducible cytokine A19 precursor	6363	-1.20	0.02586	predicted						
NR1H3	Oxysterols receptor LXR-alpha (Liver X receptor alpha)	10062	-1.23	0.00207	reported						
RRAS	Ras-related protein R-Ras precursor (p23)	6237	-1.16	0.01775	predicted						
FZD2	Frizzled-2 precursor	2535	-1.23	0.01046	predicted						
ATPAF1	ATP synthase mitochondrial F1 complex assembly factor 1 isoform 1 precursor	64756	-1.16	0.03081	predicted						
FTSJ2	Putative ribosomal RNA methyltransferase 2	29960	-1.16	0.01201	predicted						
TOB1	Tob1 protein (Transducer of erbB-2 1)	10140	-1.14	0.03696	predicted						
HspBP1	Hsp70-binding protein 1 (Heat shock protein-binding protein 1)	23640	-1.13	0.02131	predicted						
CD68	Macrosialin precursor (CD68 antigen)	968	-1.15	0.01269	predicted						
PAQR4	Progestin and adipoQ receptor family member 4	124222	-1.12	0.04328	predicted						
C14orf4	RING finger protein C14orf4	64207	-1.14	0.01352	predicted						
MYADM	Myeloid-associated differentiation marker	91663	-1.14	0.00947	predicted						
MRPL48	39S ribosomal protein L48, mitochondrial precursor	51642	-1.11	0.03522	predicted						
STAG3	Cohesin subunit SA-3	10734	-1.24	0.00449	predicted						
MARVELD1	MARVEL domain containing 1	83742	-1.18	0.01274	predicted						
PRRT2	proline-rich transmembrane protein 2	112476	-1.14	0.04240	predicted						
TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	23158	-1.18	0.00424	predicted						
FAM35A	Protein FAM35A	54537	-1.13	0.04945	predicted						
NUP62	L-amino-acid oxidase precursor	259307	-1.10	0.03627	predicted						
WTAP	Wilms' tumor 1-associating protein	9589	-1.11	0.03427	predicted						
SGK2	Serine/threonine-protein kinase Sgk2	10110	-1.11	0.02416	predicted						
CCNY	Cyclin fold protein 1 (Cyclin box protein 1)	219771	-1.09	0.04023	predicted						
DLEU1	deleted in lymphocytic leukemia,1	10301	-1.10	0.03335	predicted						
PDF	Peptide deformylase, mitochondrial precursor	64146	-1.09	0.04462	predicted						
PDF	Peptide deformylase, mitochondrial precursor	84342	-1.09	0.04462	predicted						
IQCG	IQ domain-containing protein G	84223	1.01	0.03782	predicted						
GBA2	Non-lysosomal glucosylceramidase	57704	1.12	0.02249	predicted						
TGFBR2	TGF-beta receptor type-2 precursor	7048	1.13	0.02284	predicted						
SLC25A28	Mitoferrin-2 (Mitochondrial iron transporter 2)	81894	1.14	0.00979	predicted						
NISCH	nischarin	11188	1.17	0.00321	predicted						
FASTK	Fas-activated serine/threonine kinase	10922	1.12	0.02913	predicted						
POLD4	DNA polymerase subunit delta 4	57804	1.11	0.03927	predicted						
FGR	Proto-oncogene tyrosine-protein kinase FGR	2268	1.14	0.01256	predicted						
CLDN15	Mitochondrial fission 1 protein	24146	1.13	0.02469	predicted						
GPS2	G protein pathway suppressor 2	2874	1.17	0.01149	predicted						
TMEM43	Transmembrane protein 43	79188	1.16	0.04998	predicted						
TAPBP	Tapasin precursor	6892	1.18	0.03900	predicted						
GPR174	Probable G-protein coupled receptor 174.	84636	1.12	0.03679	predicted						
LINS1	lines homolog 1	55180	1.19	0.01190	predicted						
POLG2	DNA polymerase subunit gamma 2	11232	1.19	0.00451	predicted						
LOC168455	hypothetical protein LOC168455	168455	1.19	0.00392	predicted						
HIFPH4	Putative HIF-prolyl hydroxylase PH-4	54681	1.14	0.01885	predicted						
C1orf162	Uncharacterized protein C1orf162.	128346	1.19	0.00462	predicted						
NPHP4	Nephrocystin-4 (Nephroretinin).	261734	1.23	0.00317	predicted						
OTUD5	OTU domain-containing protein 5.	55593	1.11	0.01592	predicted						
ARIH2	Protein ariadne-2 homolog	10425	1.12	0.01439	predicted						
U2AF1L4	U2 small nuclear RNA auxiliary factor 1-like 4 isoform 2	199746	1.13	0.02068	predicted						
AKAP8L	A-kinase anchor protein 8-like (	26993	1.13	0.00936	predicted						
CYP27B1	25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial precursor	1594	1.15	0.00400	predicted						
CBY1	Protein Chibby	25776	1.18	0.00107	predicted						
PPARG	Peroxisome proliferator-activated receptor gamma	5468	1.20	0.00669	predicted						
MITF	Microphthalmia-associated transcription factor	4286	1.15	0.00643	predicted						
TXNIP	Thioredoxin-interacting protein	10628	1.15	0.00553	predicted						
KIAA0467	Uncharacterized protein KIAA0467	23334	1.18	0.00250	predicted						
CXCL16	Small inducible cytokine B16 precursor	58191	1.17	0.00739	predicted						
VMO1	Vitelline membrane outer layer protein 1 homolog precursor	284013	1.20	0.00200	predicted						
GPIBb	glycoprotein Ib beta polypeptideprecursor	2812	1.18	0.02852	predicted						
INSIG1	Insulin-induced gene 1 protein	3638	1.13	0.04718	non DR1						
SCML1	Sex comb on midleg-like protein 1	6322	1.13	0.04360	predicted						
ACRC	ACRC protein	93953	1.21	0.01719	predicted						
TLR4	Toll-like receptor 4 precursor	7099	1.23	0.00938	predicted						
C15orf17	Uncharacterized protein C15orf17	57184	1.16	0.02275	predicted						
ERRFI1	ERBB receptor feedback inhibitor 1	54206	1.19	0.00438	predicted						
ZMAT1	zinc finger, matrin type 1 isoform 1	84460	1.18	0.00535	predicted						
TESK2	Dual specificity testis-specific protein kinase 2	10420	1.20	0.00694	predicted						
AKT1S1	Proline-rich AKT1 substrate 1	84335	1.23	0.00185	predicted						
BACH1	Transcription regulator protein BACH1	571	1.19	0.00544	predicted						
G0S2	Putative lymphocyte G0/G1 switch protein 2	50486	1.25	0.00540	predicted						
MBOAT5	Membrane-bound O-acyltransferase domain-containing protein 5	10162	1.26	0.00282	predicted						
ABCA1	ATP-binding cassette sub-family A member 1	19	1.32	0.00030	reported						
CD36	Leukocyte differentiation antigen CD36	948	1.29	0.03259	predicted						
CYP1A1	Cytochrome P450 1A1	1543	1.26	0.02995	predicted						
CLCF1	Cardiotrophin-like cytokine factor 1 precursor	23529	1.15	0.04082	predicted						
CAPN3	Neutral alpha-glucosidase C	825	1.18	0.00492	predicted						
SMAD3	Mothers against decapentaplegic homolog 3	4088	1.23	0.00127	predicted						
MMP19	Matrix metalloproteinase-19 precursor	4327	1.22	0.01219	predicted						
CPT1B	Carnitine O-palmitoyltransferase I, muscle isoform	1375	1.23	0.00272	predicted						
ATHL1	acid trehalase-like 1	80162	1.27	0.00362	predicted						
AP1G2	Junctophilin-4 (Junctophilin-like 1 protein).	8906	1.19	0.00136	predicted						
USP52	PAB-dependent poly(A)-specific ribonuclease subunit 2	9924	1.27	0.00012	predicted						
TSPYL2	TSPY-like 2	64061	1.21	0.00472	predicted						
LOC54540	hypothetical protein LOC54540	54540	1.27	0.00049	predicted						
PLA2G4B	Cytosolic phospholipase A2 beta	8681	1.16	0.02102	predicted						
IL11RA	Interleukin-11 receptor alpha chain precursor	3590	1.19	0.02288	predicted						
SLC25A34	solute carrier family 25, member 34	284723	1.22	0.00967	predicted						
VEGFA	Vascular endothelial growth factor A precursor	7422	1.22	0.00807	predicted						
TANFKBH	T-cell activation NFKB-like protein	84807	1.29	0.00236	predicted						
TMEM185A	Transmembrane protein 185A	84548	1.25	0.01367	predicted						
NXF1	Nuclear RNA export factor 1	10482	1.35	0.00047	predicted						
CSAD	Cysteine sulfinic acid decarboxylase	51380	1.37	0.00238	predicted						
CYorf15B	lipopolysaccaride-specific response 5-like protein	84663	1.42	0.00046	predicted						
TPP1	Tripeptidyl-peptidase 1 precursor	1200	1.40	0.03811	predicted						
SFRS16	Splicing factor, arginine/serine-rich 16	11129	1.44	0.02157	predicted						
TMEM135	Transmembrane protein 135	65084	1.66	0.00142	predicted						
ACAA2	3-ketoacyl-CoA thiolase, mitochondrial (Acetyl-CoA acyltransferase)	10449	1.46	0.00002	predicted						
SLC25A20	Mitochondrial carnitine/acylcarnitine carrier protein (Carnitine/acylcarnitine translocase)	788	1.52	0.00000	reported						
ACADVL	Very-long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	37	2.06	0.00000	predicted						
ADFP	Adipophilin (Adipose differentiation-related protein)	123	3.78	0.00000	reported						
PDK4	Pyruvate dehydrogenase kinase 4	5166	4.72	0.00000	reported						
FABP4	Fatty acid-binding protein, adipocyte	2167	13.89	0.01455	reported						

subsequent transcription factor binding site search with Genomatix software was performed. The network analysis showed that, besides the transcription factor PPAR, the transcription factors NFkB, JUN, TP53, SP1 and CTNNB1 were also directly linked to at least 10 genes from the list of 1,373 genes, The subsequent search for binding sites resulted in an additional 122 genes that could be linked to a PPRE and revealed that another 371 genes could be linked to at least one of the other selected transcription factors (**Additional file**).

To obtain a selection of robust responding genes upon activation of PPARα with WY14.643, genes were selected that were more than 10% up or down regulated in all donors . This resulted in a list of 58 genes, including several known PPARα target genes (*ADFP, PDK4, SLC25A20*) (**Figure 4**), with a main function in fatty acid β-oxidation. Remarkably, only 16 % of the genes in this list contained a predicted or reported PPRE.

Gene Name	Gene Description	Mean FC	p value	Entrez ID	PPRE	SLR A	SLR B	SLR C	SLR D	SLR E	SLR F
ADFP	Adipose differentiation-related protein	3.78	2.86E-10	123	reported						
PDK4	Pyruvate dehydrogenase kinase isozyme 4	4.72	6.16E-07	5166	reported						
ACADVL	Very-long-chain specific acyl-CoA dehydrogenase	2.06	1.05E-07	37	predicted						
LY6G5B	Casein kinase II subunit beta	1.88	4.82E-05	58496	-						
LOC283874	Hypothetical protein FLJ20393	1.66	8.72E-05	283874	-						
SLC25A20	Mitochondrial carnitine/acylcarnitine carrier protein	1.52	8.68E-07	788	reported						
IMPA2	Inositol monophosphatase 2	1.47	1.01E-05	3613	-						
C21orf7	TAK1-like protein.	1.40	1.79E-04	56911	-						
ST14	Suppressor of tumorigenicity protein 14	1.60	2.84E-05	6768	-						
HS3ST1	Heparan sulfate glucosamine 3-O-sulfotransferase 1	1.51	2.55E-04	9957	-						
ETFDH	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	1.42	1.32E-04	2110	-						
MICALL2	MICAL-like protein 2.	1.36	1.65E-04	79778	-						
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.60	2.35E-04	1326	-						
TMEM135	Transmembrane protein 135.	1.66	1.42E-03	65084	predicted						
ACAA2	3-ketoacyl-CoA thiolase, mitochondrial	1.46	2.37E-05	10449	predicted						
BZRAP1	Peripheral-type benzodiazepine receptor-associated protein 1	1.36	2.93E-05	9256	-						
ABCC3	Canalicular multispecific organic anion transporter 2	1.34	9.88E-05	8714	-						
LOC158830	similar to Ab2-183	1.41	1.51E-04	158830	-						
C20orf119	Polyadenylate-binding protein 1-like.	1.40	2.80E-04	80336	-						
PPP1R3E	Homeobox and leucine zipper protein Homez	1.31	1.29E-04	90673	-						
PILRB	paired immunoglobulin-like type 2 receptor beta isoform b	1.32	1.58E-04	29990	-						
HBEGF	Heparin-binding EGF-like growth factor	1.36	7.70E-05	1839	-						
CXCL2	Macrophage inflammatory protein 2-alpha	1.31	6.44E-04	2920	-						
CCDC17	coiled-coil domain containing 17	1.25	6.35E-04	149483	-						
CYorf15B	lipopolysaccaride-specific response 5-like protein	1.42	4.61E-04	84663	predicted						
NLRC4	Caspase recruitment domain-containing protein 12	1.36	3.15E-04	58484	-						
USP52	PAB-dependent poly(A)-specific ribonuclease subunit 2	1.27	1.17E-04	9924	predicted						
FNBP4	formin binding protein 4	1.29	3.83E-04	23360	-						
COG3	Conserved oligomeric Golgi complex component 3	1.23	6.07E-04	83548	-						
CD300A	CMRF35-H antigen precursor	1.26	5.91E-04	11314	-						
ABCC5	Multidrug resistance-associated protein 5	1.24	9.97E-04	10057	-						
CREBZF	CREB/ATF bZIP transcription factor	1.26	2.51E-03	58487	-						
MPP7	palmitoylated membrane protein 7	1.24	8.44E-04	143098	-						
ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.23	1.38E-03	1891	-						
DEADC1	deaminase domain containing 1	1.20	5.78E-04	134637	-						
CLK2	Dual specificity protein kinase CLK2	1.19	1.52E-03	1196	-						
TTRAP	TRAF and TNF receptor-associated protein	1.23	1.15E-03	51567	-						
SPG7	Paraplegin	1.19	9.28E-04	6687	-						
MTX3	metaxin 3	1.14	5.25E-03	345778	-						
CCDC130	Coiled-coil domain-containing protein 130	1.20	1.18E-03	81576	-						
CBY1	Protein Chibby	1.18	1.07E-03	25776	predicted						
SYTL1	Synaptotagmin-like protein 1	1.17	1.75E-03	84958	-						
C6orf70	Uncharacterized protein C6orf70	1.17	2.19E-03	55780	-						
GPNMB	Transmembrane glycoprotein NMB	-1.41	9.23E-06	10457	-						
EMR1	EGF-like module-containing mucin-like hormone receptor-like 1	-1.38	1.30E-05	2015	-						
ANXA3	Annexin A3	-1.42	3.57E-05	306	-						
TMEM176A	Transmembrane protein 176A	-1.29	4.95E-05	55365	-						
CAPG	Macrophage-capping protein	-1.30	1.06E-04	822	-						
DPYSL2	Dihydropyrimidinase-related protein 2	-1.38	6.02E-05	1808	-						
IGSF6	immunoglobulin superfamily, member 6	-1.31	2.52E-04	10261	-						
IFI6	Interferon-induced protein 6-16	-1.32	3.05E-04	2537	-						
ADORA3	Adenosine A3 receptor	-1.34	8.81E-04	140	-						
C1orf115	Uncharacterized protein C1orf115.	-1.23	2.82E-04	79762	-						
TRIP10	Cdc42-interacting protein 4	-1.20	9.05E-04	9322	-						
FPR1	fMet-Leu-Phe receptor	-1.22	5.83E-04	2357	-						
TNFRSF8	Tumor necrosis factor receptor superfamily member 8	-1.18	2.37E-03	943	-						
GAS2L1	GAS2-like protein 1	-1.18	1.18E-03	10634	-						
C1orf163	Hcp beta-lactamase-like protein C1orf163.	-1.18	1.43E-03	65260	-						

Figure 4 (left page) Heat map of genes changed more than 10% in all individuals after incubation with WY14,643. Red indicates up-regulation and green indicates down-regulation. SLR, signal log ratio; PPRE, peroxisome proliferator response element

To validate our data observed with microarray analyzes a selection of genes changed in the microarray analyzes was also measured with quantitative real time PCR (Q-PCR). In concordance with our microarray results, Q-PCR analyzes resulted in similar changes in expression of all genes analyzed (**Figure 5**).

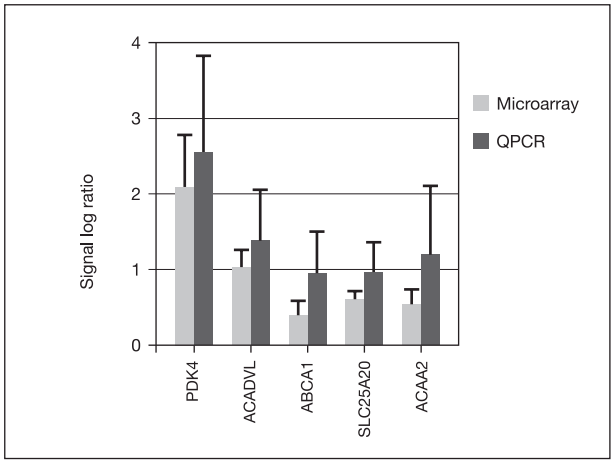

Figure 5 Mean gene expression changes of microarray and quantitative real time PCR analysis (Q-PCR) of six genes after incubation with WY14,643. Error bars indicated standard deviations. *PDK4, Pyruvate dehydrogenase kinase 4; ACADVL, acyl-Coenzyme A dehydrogenase, very long chain; ABCA1, ATP binding cassette transporter 1; SLC25A20, carnitine-acylcarnitine translocase; ACAA2, acetyl-Coenzyme A acyltransferase 2*.

PPARα regulation in PBMCs during fasting

Figure 6 shows the genes changed upon 24 hours fasting in healthy human volunteers with the number of genes that contain a PPRE. Comparison of gene expression profiles of PBMCs incubated with the PPARα ligand WY14,643 and fasted for 24 hours resulted in an overlap of 238 genes, indicating that around 14 % of the genes changed during fasting are regulated by PPARα (**Figure 7**). Pathway analysis showed that these 238 genes were primarily involved in fatty acid metabolism. We found no overlap in pathways involved in amino acid

metabolism. Exploration of the genes involved in fatty acid metabolism showed that fatty acid β-oxidation was specifically regulated, both in WY14,643 incubated cells and in PBMCs isolated after fasting (data not shown).

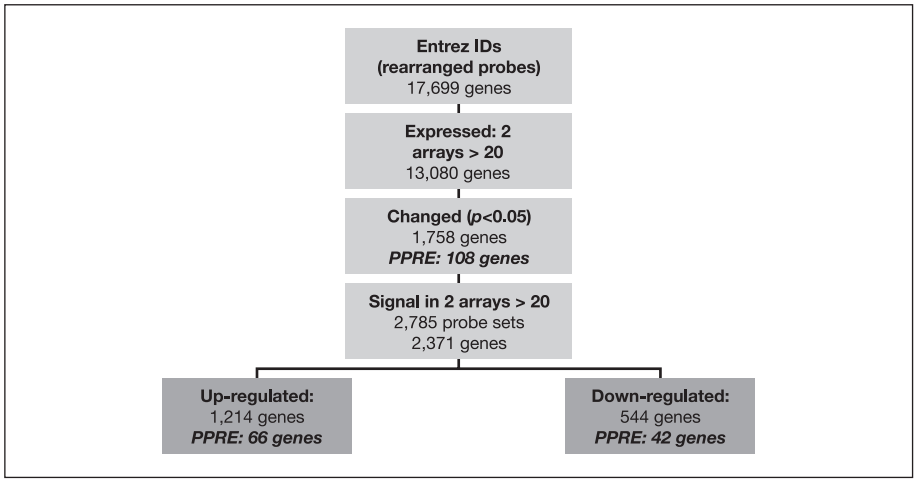

Figure 6 Flow chart of the followed gene selection procedure after microarray analysis of PBMC of three 24 hour fasted subjects. PPRE; number of genes containing a peroxisome proliferator response element according to Lemay et al. Data from this fasting study was published previously[67], but has been used here after applying a different annotation procedure.

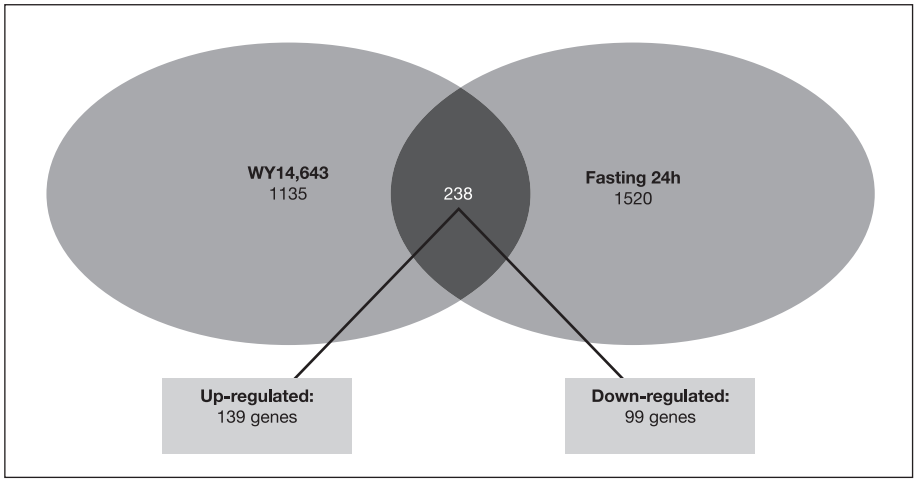

Figure 7 Venn diagram of overlap between genes changed upon WY14,643 incubation and after 24 hours fasting.

Discussion

In the present study, we showed that activation of the nuclear receptor PPARα in peripheral blood mononuclear cells results in a considerable change in gene expression profiles, as 10.5% of the genes expressed exhibited altered gene expression levels after incubation with the specific PPARα agonist WY14,643. The main function of PPARα in PBMCs appeared to be the regulation of fatty acid β-oxidation and other lipid metabolism related functions, which is in line with results from mice studies in liver[36] and intestine[73], and human cell line studies[74, 75]. Moreover, the observed down-regulation of amino acid metabolism has been shown before in liver in studies comparing wild type mice to the PPARα knock out mouse model[63].

Besides the possible roles of PPARα in PBMCs, this study also demonstrates strong individual variability between the subjects in gene expression responses to activation with WY14,643. It appears that each donor has its own specific gene expression profile response to PPARα activation, which results in distinct differences in the expression of certain genes after WY14,643 incubation. Beck *et al.* also reported differences in responsiveness in gene expression between individuals, after incubation of endothelial cells with LPS. However, endothelial cell cultures were already divided beforehand into type I or type II responders based on their LPS mediated IL8 production[76]. In another study, incubation of cultured macrophages with oxidized low-density lipoprotein resulted in a person-specific inflammatory gene expression response that could be correlated to changes in gene expression of scavenger receptors[77]. However, we did not find a correlation between basal *PPARα* expression or changes in *PPARα* expression and the observed variation in gene expression changes. In addition, the differences observed are probably not caused by the nutritional status of the subjects at baseline, as we did not observe differences in expression changes of selected PPARα target genes between the postprandial and the fasted state of PBMCs incubated with WY14,643. However, it should be noted here that only four subjects were studied. A reason for the difference in response of the donors in the first study could be genetic variation, such as single nucleotide polymorphisms (SNPs) in the *PPARα* gene, its target genes or PPARα co-factors involved in activation of gene transcription. Furthermore, epi-genetic variation such as methylation status of the PPARα promoter or its target genes may have caused between-subject differences in gene expression levels. Additional studies

are required to elucidate whether gene expression profiles can be clustered in different response profiles, simplifying the identification of factors responsible for these individual responses. With respect to personalized nutrition these individual responses are of great interest as it can be expected that nutrients such as fatty acids can induce similar variations in response as WY14,643, which in the end might lead to personalized dietary advice.

The PPRE analyzes of the genes changed showed that approximately 8% of the genes changed after incubation with the PPARα ligand WY14,643 contained a predicted or reported PPRE, using the list as described by Lemay et al[70]. However, Lemay *et al.* report that they tolerate a low false-positive, and a high (60%) false-negative rate, suggesting that their list of PPREs is far from complete. Our additional transcription factor binding site analysis increase the number of genes that contain a PPRE to a total of 17% of the genes changed. A network search showed that, besides PPAR, five other transcription factors were involved in direct regulation of at least 10 out of the 1,373 changed genes. Interestingly, all these transcription factors are known to be affected by PPARα activation[78-82]. Transcription factor binding site analysis revealed that, out of the changed genes that did not contain a PPRE, 27% contained a binding site for at least one of the other five selected transcription factors These genes appear not to be regulated by PPARα directly, but indirectly, via these other transcription factors, a mechanism which has been suggested before[83, 84]. The role of PPARα in this respect seems to be extensively larger than expected based on the outcome of PPRE analyzes alone.

An interesting observation is the decrease in expression level of genes containing a PPRE. Activation of PPARα by a ligand may result in a negative regulation of genes by means of transrepression as has been reported in several studies and reviewed by Ricote and Glass (2007)[23]. This transrepression, however, does not require the presence of PPREs in the promoter regions of the target genes. Apparently, negative regulation of these genes, regardless of its mechanism, is stronger than the transcriptional activation of PPARα. Previously, Degenhardt et al. (2006) also showed down regulation of an insulin-like growth factor-binding protein gene (IGFBP-6) that contained a predicted PPRE, in response to the presence of a PPARα ligand[85].

The overlap in gene expression profiles between fasting and incubation with WY14,643 shows that PPARα in PBMCs carries out a substantial part of its

function during fasting, when concentrations of its natural ligands, free fatty acids, are elevated in the blood. The main role of PPARα in PBMCs during fasting is fatty acid β-oxidation, most likely to cope with the reduced availability of glucose for utilization in energy production and the increase of fatty acids.

Direct comparison between the two array analysis should be examined with care, since the two studies are distinctly different in set-up. The fasting intervention study was conducted *in vivo*, while the WY14,643 incubation experiments were performed *ex vivo*. Moreover, fasting involves many more changes in physiology, apart from the before-mentioned increase in plasma free fatty acids, including changes in plasma insulin, glucose and leptin concentrations. The PPARα ligand incubations were set-up to elucidate the specific effects of activation of one nuclear factor, controlling for all other parameters.

Summarizing, this study gives us valuable information on the extent of the effect of PPARα activation, during fasting and in general, on human PBMC gene expression. It also shows that persons respond differently to PPARα activation with respect to their gene expression changes, indicating a possible person-specific nutrient response. It seems justified to conclude that human PBMCs are a suitable model to study changes in PPARα activation. This opens up the possibilities for more specific PPARα signaling studies in healthy humans using these relatively easily obtainable blood cells.

Authors' contributions

MB collected and analyzed the data and wrote the manuscript. LAA an MM participated in critical revising of the manuscript. None of the authors has a personal or financial conflict of interest.

Acknowledgements

The authors would like to thank Mechteld Grootte Bromhaar for conducting the microarray processing. This study was supported by the Dutch Dairy Association (Zoetermeer, The Netherlands).

Additional file can be found online:

www.biomedcentral.com/content/supplementary/1471-2164-9-262-s1.pdf

Presence of transcription factor binding sites in the genes changed in PBMC after incubation with WY14,643. Transcription factors were selected if they directly

affected at least 10 genes that were changed after WY14,643 incubation, in a network search using BiblioSphere (Genomatix). FC, fold change; PPRE (Lemay), peroxisome proliferator response element according to Lemay *et al.*[70]; NFkB, Nuclear factor kappa B binding site; JUN, Jun oncogene binding site; TP53, Tumor protein 53 binding site; SP1, Specificity protein 1 binding site; CTNNB1, catenin beta 1 binding site. Red indicates up regulated, green indicates down regulated.

Chapter 4

Fish oil supplementation induces anti-inflammatory gene expression profiles in human blood mononuclear cells

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Submitted for publication
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Abstract

Gene expression profiles of peripheral blood mononuclear cells (PBMCs) have been shown to reflect the pathological state of an individual, and we recently showed that these cells can also reflect physiological changes. However, little is known about the effects of nutrition on PBMC gene expression profiles. Nutrients such as polyunsaturated fatty acids (PUFAs) are known to elicit effects through gene transcription changes via activation of transcription factors, such as peroxisome proliferator-activated receptors. Therefore, we examined the effects of supplementation with the PUFAs EPA/DHA on whole genome PBMC gene expression profiles in healthy Dutch elderly, participating in a double-blind trial. Individuals were randomly assigned to consume 1.8 gram/day of EPA/DHA, 0.4 gram/day of EPA/DHA or high oleic acid sunflower oil (HOSF) in capsules for 26 weeks. Microarray analysis was performed on PBMC RNA from subjects who received high EPA/DHA (n=23) or HOSF capsules (n=25). Q-PCR was performed on the high EPA/DHA group (n=36), low EPA/DHA group (n=37) and the HOSF group (n=37). High EPA/DHA intake resulted in a change of 1040 genes and HOSF intake resulted in 298 genes changed. EPA/DHA intake showed lowered expression of genes in inflammatory and atherogenic related pathways, such as NF-κB signaling, eicosanoid synthesis, scavenger receptors activity, adipogenesis and hypoxia signaling. These results reveal that intake of EPA/DHA for 26 weeks alters gene expression profiles of PBMCs to a more anti-inflammatory status. Moreover, this nutrigenomics study demonstrates that PBMC profiling can not only reflect pathological conditions or physiological state, but can also mimic nutritional changes.

Introduction

The last few years there has been increasing interest in the use of gene expression profiles of peripheral blood mononuclear cells (PBMCs) as diagnostic biomarkers for the pathological state[8, 86]. The reason for this interest is the relatively simple accessibility of blood cells compared with other tissues in humans. We recently showed that, besides their usefulness to reveal disease states, these cells can also reflect physiological changes, such as fasting[67]. However, although some studies have been performed recently[87], still little is known about the effects of nutrition on PBMCs gene expression profiles. Nutrients known to elicit effects on gene expression are fatty acids, which act mainly through activation of transcription factors, such as peroxisome proliferator-activated receptors (PPARs)[88]. These nuclear receptors are known to up- and down-regulate expression of genes involved in lipid metabolism and inflammation, respectively. Three types of PPARs have been identified; alpha, gamma and delta/beta[24], which all have specific roles in different tissues. We have recently shown that PPARα has a functional role in human PBMCs[89.] The strongest natural ligands for PPARs are long chained polyunsaturated fatty acids (PUFAs), such as the n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)[90], which are abundantly present in fatty fish. N-3 PUFA are therefore good candidates to explore the effects of nutrition on PBMC gene expression profiling. We examined the effects of EPA/DHA supplementation for 26 weeks on whole genome PBMC gene expression profiles in a Dutch elderly population and compared the effects to a control supplementation of high oleic acid sunflower oil (HOSF).

Methods

Subjects

Healthy men and women of 65 years and over were screened and recruited according to the following exclusion criteria: current or recent (<4 weeks) use of fish oil supplements or intake of fish more than four times per week or more than 800 mg of EPA-DHA from fish per day as estimated by a fish consumption questionnaire, serious liver disease, consumption of more than 4 glasses of alcohol per day, unable to participate as judged by the responsible medical physician, allergy to fish(oil), swallowing problems, or participation in another clinical trial less than 2 months before the start of the trial or at the same time. Additionally, compliance to capsule use during a 2-week placebo run-in period had to be at least 80% on basis of self-report. 302 Subjects were included in the study and detailed baseline characteristics of these participants have been described elsewhere[91]. From these participants, 111 subjects were randomly included in the present study. All subjects gave written informed consent to participate in the study and the study protocol was approved by the Medical Ethical Committee of Wageningen University, the Netherlands.

Study design

Subjects were randomly allocated to receive a daily dose of fish oil containing either a low dose of 0.4 g EPA/DHA, a high dose of 1.8 g EPA/DHA, or a high oleic acid sunflower oil (HOSF) for a period of 26 weeks (Lipid Nutrition/ Loders Croklaan, Wormerveer, the Netherlands). The oils were administered in six soft gelatin capsules daily, each containing 900 mg oil and 2.7 mg tocopherol as antioxidant (Banner Pharmacaps Europe B.V., Tilburg, the Netherlands). The fatty acid composition of the capsules was measured in 20 capsules taken at regular times during the study.

Blood sampling and PBMC isolation

Fasting venous blood samples were collected at baseline and after 26 weeks of intervention. A blood sample for determination of n-3 PUFAs was collected into 10 ml EDTA-vacutainers, immediately placed on ice, centrifuged at 2000 g at a temperature of 4 °C and then stored at -80 °C until laboratory analyses. N-3 PUFAs in plasma cholesteryl esters were determined as described previously[92]. For PBMC isolation 4 ml of blood was collected in BD Vacutainer Cell Preparation

Tubes with sodium citrate (BD, Breda, The Netherlands). PBMCs were isolated immediately after blood collection according to the manufacturer's manual.

Total RNA isolation

PBMC RNA was isolated from all PBMC samples using Qiagen RNeasy Micro kit (Qiagen, Venlo, the Netherlands). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (Nanodrop technologies, Wilmington, Delaware USA) and integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, UK).

Microarray processing

PBMC samples from 77 subjects yielded enough RNA to perform microarray analysis. De-blinding revealed that 25 subjects were in the high EPA/DHA group, 26 in the low EPA/DHA group and 26 in the HOSF group. Microarray analysis was performed on baseline samples and samples after 26 weeks of intervention in the high EPA/DHA group and the HOSF group. Total RNA from PBMCs of these 51 subjects was labeled using a one-cycle cDNA labeling kit (MessageAmp™ II-Biotin *Enhanced* Kit, Ambion, Inc.) and hybridized to human whole genome NuGO GeneChip arrays encoding 17,699 genes, designed by the European Nutrigenomics Organisation (NuGO) and manufactured by Affymetrix (Affymetrix Inc, Santa Clara, CA). Sample labeling, hybridization to chips and image scanning was performed according to the manufacturer's GeneChip Expression Analysis Technical Manual (Affymetrix).

Microarray analysis

Microarrays from three subjects, 2 from the high EPA/DHA group and 1 from the HOSF group, were excluded because they did not fulfill our quality control criteria. Microarrays were analyzed using the reorganized oligonucleotide probes as described by Dai *et al* (2005)[68]. Expression values were calculated using the Robust Multichip Average (RMA) method. RMA signal value estimates are based on a robust average of background corrected perfect match intensities and normalization was performed using quantile normalization[42]. Only genes with normalized signals higher than 20 on at least 10 arrays were defined as 'expressed' and selected for further analysis. Genes were defined as 'changed' when comparison of the normalized signal intensities showed a false discovery

rate Q-value[93] lower than 0.05 in a two-tailed paired *t* test with Bayesian correction (Limma) (69). Pathway analysis was performed using GenMAPP 2.1, Ingenuity Pathway Analysis 5.5 (IPA), and Gene Set Enrichment Analysis 2.0.1 (GSEA)[94].

cDNA synthesis and real time PCR

RNA from all subjects was reverse transcribed with use of a cDNA synthesis kit (Promega, Leiden, the Netherlands). Standard quantitative real-time polymerase chain reaction (Q-PCR) was performed with use of Platinum Taq DNA polymerase and SYBR Green on an iCycler PCR machine (Bio-Rad Laboratories BV) and duplicated at least twice. Primer sequences used in the PCRs were chosen based on the sequences available in PRIMERBANK (http://pga.mgh.harvard.edu/ primerbank/index.html). Q-PCR data were normalized by measuring cycle threshold ratios between candidate genes and a housekeeping gene, human ribosomal protein LP0.

Statistical Analysis

Differences between baseline and end values of plasma metabolites and Q-PCR results were tested using a paired t-test and differences between groups were tested using ANOVA in the statistical package SPSS 12.0.1 (Chicago, Illinois). Statistical significance was accepted at $p<0.05$. Pathways were defined as significantly changed when $p<0.05$ (IPA and GSEA) or the z-score was higher than 1 (GenMAPP).

Results

Subject characteristics

From the 302 participants who were enrolled in the study, blood samples where randomly collected from 111 healthy men and woman varying in age from 66 to 80 years old. A subset of these samples was used to perform microarray hybridizations. The baseline characteristics of the participants are shown in **Table 1**. No significant differences were present between the intervention groups, both for the whole group, and the subgroup on which the microarrays were performed. To confirm the amount of the various fatty acids the participants consumed daily, the fatty acid composition of the capsules was measured (**Table 2**). The high dose of EPA/DHA provided 1093 ± 17 mg EPA (mean ± SD) and 847

± 23 mg of DHA daily and the low dose of EPA/DHA provided 226 ± 3 mg EPA and 176 ± 4 mg of DHA daily.

Table 1 Baseline characteristics of array and QPCR subjects.

	High EPA/DHA		Low EPA/DHA	HOSF	
	QPCR subjects	Array subjects	QPCR subjects	QPCR subjects	Array subjects
Subjects (n)	36	23	37	38	25
Gender (M/F)	21/15	15/8	20/17	25/13	15/10
Age (years)	70.3 (67-76)	69.9 (67-76)	70.5 (66-79)	70.9 (66-80)	70.4 (67-77)
Smokers (n)	1	0	4	3	2
Weight (kg)	76.6 (60.1-106.7)	78. (60.1 – 106.7)	75.3 (47.6-101.8)	79.1 (59.1-108.6)	81.1 (59.1 – 109.5)
Height (m)	1.71 (1.58-1.86)	1.72 (1.58 – 1.86)	1.70 (1.53-1.87)	1.73 (1.57-1.87)	1.72 (1.57 – 1.87)
BMI (kg/m^2)	26.2 (21.12-33.6)	26.5 (21.7 – 33.6)	25.8 (19.9-34.9)	26.5 (20.2-37.2)	27.6 (20.2 – 42.3)
TG (mmol/L)	1.14 (0.4-3.3)	1.02 (0.4-2.0)	1.17 (0.2-3.8)	1.18 (0.4-2.7)	1.20 (0.5-2.7)
FFA (mmol/L)	0.38 (0.12-0.71)	0.35 (0.12-0.59)	0.36 (0.08-0.99)	0.34 (0.06-0.98)	0.36 (0.06-0.98)
EPA (mass%)	1.26 (0.4-4.4)	1.44 (0.6-4.4)	1.43 (0.4-5.0)	1.30-(0.4-8.5)	1.38 (0.4-8.5)
DHA (mass%)	0.62 (0.3-1.1)	0.65 (0.3-1.1)	0.66 (0.2-1.6)	0.64 (0.4-1.3)	0.63 (0.4-1.3)

Table 2 Composition of the capsules (%). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

		High EPA/DHA	Low EPA/DHA	HOSF
HOSF		22.08	10.40	8.46
MUFA		14.73	67.97	80.53
c18:1n-9	Oleic acid	4.66	64.38	79.63
PUFA		53.39	20.23	11.02
c18:2n-6	Linoleic acid	0.74	9.13	10.81
c20:5n-3	EPA	21.40	4.49	0.00
c22:6n-3	DHA	16.44	3.46	0.00

Plasma measurements

To check compliance of individual intake, plasma fatty acid composition was determined in cholesteryl esters at baseline and after 26 weeks (**Figure 1A**). Both EPA and DHA were significantly elevated (p<0.01) in the low and high EPA/DHA groups compared with baseline. Plasma EPA concentrations showed a mean increase of 348% in the high EPA/DHA group and a mean increase of 83% in the low EPA/DHA group. Mean plasma DHA concentration increased with 97% and 31%, respectively. No changes were observed in the group that consumed the HOSF capsules. Plasma triglyceride concentrations were significantly decreased after 26 weeks in the high EPA/DHA group (p<0.01), and not in the low EPA/DHA

or HOSF group (**Figure 1B**). Plasma free fatty acids (FFA) were also significantly decreased after 26 weeks in the high EPA/DHA group (p<0.01), and not in HOSF group. The FFA in the low EPA/DHA showed a tendency to decrease, although this was not statistically significant (p=0.06) (**Figure 1C**).

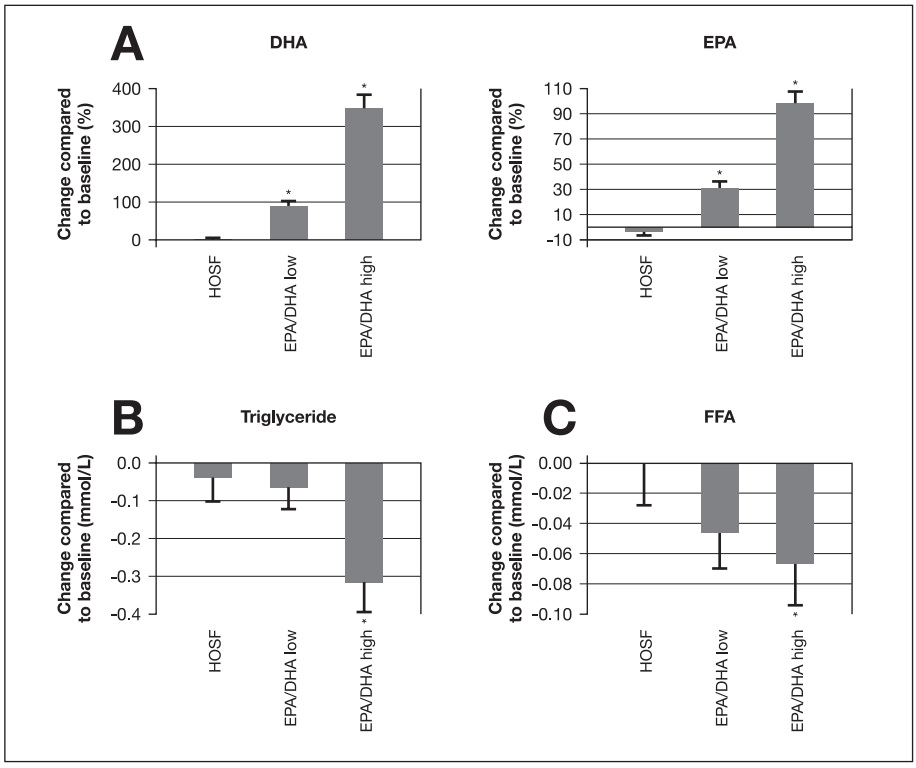


Figure 1 Changes in plasma lipid metabolites after intake of EPA/DHA or HOSF capsules for 26 weeks compared with baseline in all 111 participants. A) Plasma cholesterylesters EPA and DHA B) Plasma triglyceride concentrations C) Plasma total free fatty acid concentrations. Values are mean ±SEM, * p<0.01 compared with baseline.

Microarray analysis

Microarray hybridization was performed on PBMC RNA that was collected at baseline and after 26 weeks of supplementation from 25 subjects from the high EPA/DHA group and 26 subjects from the HOSF group. Two arrays in the high EPA/DHA group and one array in the HOSF group did not pass the quality control criteria. Changes in gene expression were determined by comparison of the microarray results of the samples after 26 weeks of intervention with microarray results of the baseline samples, in both intervention groups. From the 17,699 genes present on the microarray 12,256 genes were defined as expressed in PBMCs (**Figure 2**). Consumption of 1.8 g of EPA/DHA daily for 26 weeks resulted in gene expression changes of 1040 genes, whereas consumption of the HOSF capsules resulted in changes of 298 genes (**Figure 2**). Of these genes, 140 genes overlapped between the groups, resulting in 900 uniquely changed genes in the EPA/DHA group. Except for one gene, the direction of change of the overlapping genes was the same in the EPA/DHA and the HOSF group (data not shown).

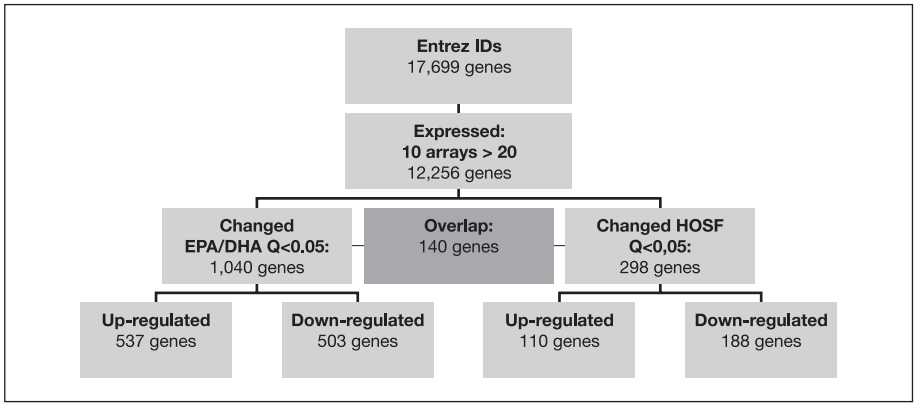
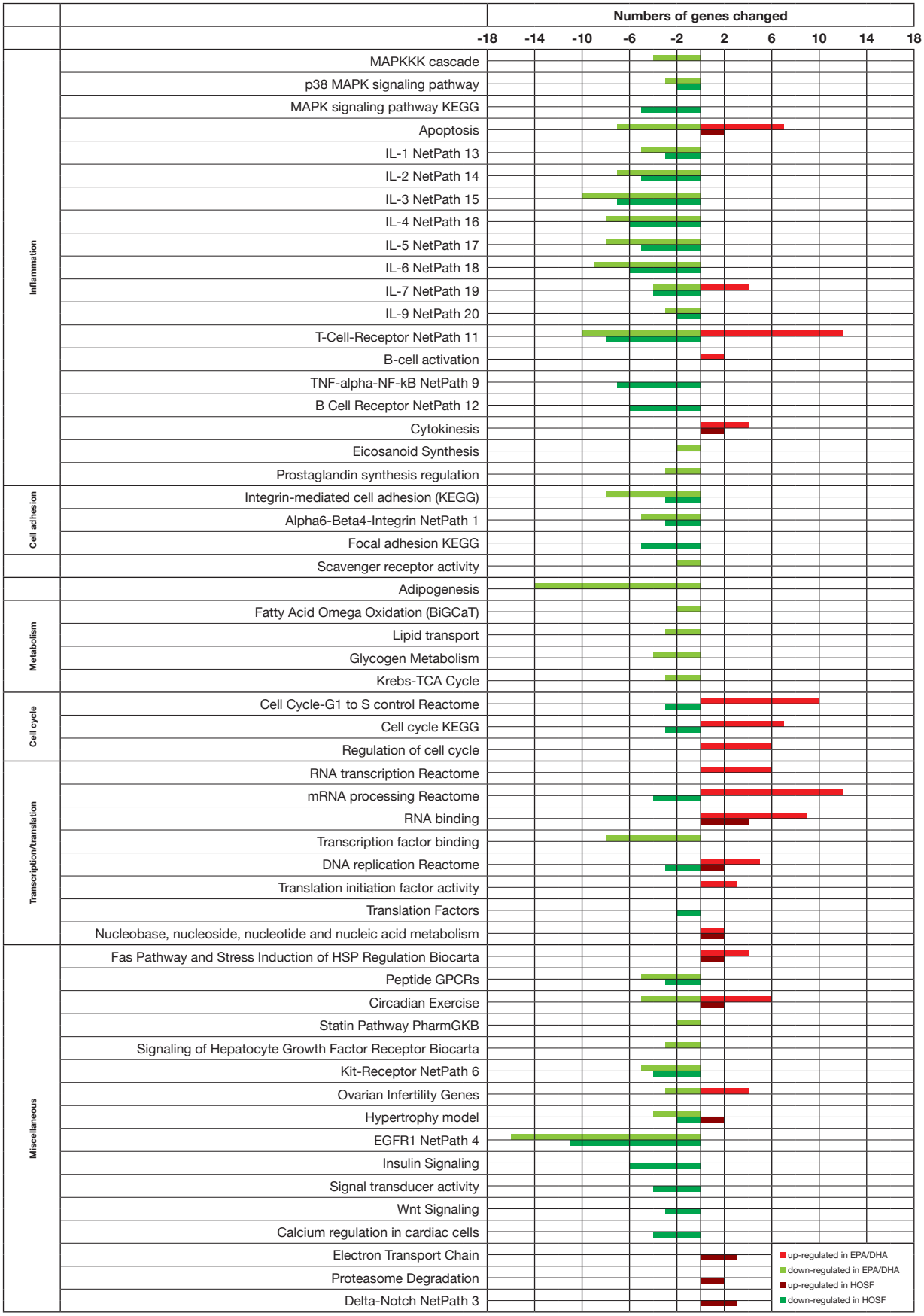


Figure 2 Flow chart of gene selection and number of genes changed in the microarray analysis. Q, false discovery rate Q value.

Pathway analysis

To determine the role of the genes that changed upon EPA/DHA supplementation in pathways, signaling routes or networks, we performed pathway analysis on all data. GenMAPP analysis revealed that supplementation with a high dose of EPA/DHA for 26 weeks significantly decreased expression of genes involved in inflammatory pathways, such as eicosanoid synthesis, interleukin signaling and


Numbers of genes changed
-18
-14
-10
-6
-2
2
6
10
14
18
Inflammation
MAPKKK cascade
p38 MAPK signaling pathway
MAPK signaling pathway KEGG
Apoptosis
IL-1 NetPath 13
IL-2 NetPath 14
IL-3 NetPath 15
IL-4 NetPath 16
IL-5 NetPath 17
IL-6 NetPath 18
IL-7 NetPath 19
IL-9 NetPath 20
T-Cell-Receptor NetPath 11
B-cell activation
TNF-alpha-NF-kB NetPath 9
B Cell Receptor NetPath 12
Cytokinesis
Eicosanoid Synthesis
Prostaglandin synthesis regulation
Cell adhesion
Integrin-mediated cell adhesion (KEGG)
Alpha6-Beta4-Integrin NetPath 1
Focal adhesion KEGG
Scavenger receptor activity
Adipogenesis
Metabolism
Fatty Acid Omega Oxidation (BiGCaT)
Lipid transport
Glycogen Metabolism
Krebs-TCA Cycle
Cell cycle
Cell Cycle-G1 to S control Reactome
Cell cycle KEGG
Regulation of cell cycle
Transcription/translation
RNA transcription Reactome
mRNA processing Reactome
RNA binding
Transcription factor binding
DNA replication Reactome
Translation initiation factor activity
Translation Factors
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism
Miscellaneous
Fas Pathway and Stress Induction of HSP Regulation Biocarta
Peptide GPCRs
Circadian Exercise
Statin Pathway PharmGKB
Signaling of Hepatocyte Growth Factor Receptor Biocarta
Kit-Receptor NetPath 6
Ovarian Infertility Genes
Hypertrophy model
EGFR1 NetPath 4
Insulin Signaling
Signal transducer activity
Wnt Signaling
Calcium regulation in cardiac cells
Electron Transport Chain
Proteasome Degradation
Delta-Notch NetPath 3
up-regulated in EPA/DHA
down-regulated in EPA/DHA
up-regulated in HOSF
down-regulated in HOSF

MAP kinase signaling (**Figure 3**). In addition, a decrease in gene expression in pathways related to atherosclerotic processes, such as cell adhesion, scavenger receptor activity and adipogenesis was observed. Other pathways negatively regulated by EPA/DHA supplementation were transcription factor binding and the cancer signaling pathway EGFR1 NetPath 4. Pathways that were up-regulated upon EPA/DHA supplementation were cell cycle regulation and RNA transcription, processing and binding.

Figure 3 (left page) Ranking of differentially expressed pathways in PBMC after 26 week supplementation of high EPA/DHA or HOSF capsules in elderly individuals. Pathway analysis was performed with the program GenMAPP. Pathways included had a z-score of at least 1 and had at least 2 genes changed.

Analyses with Ingenuity pathway analysis (IPA) revealed similar decreases in inflammatory signaling routes, such as IL6 and MAP kinase, with additional signaling routes such as NF-κB and Toll-like receptor signaling. IPA also showed a decrease in PPAR signaling, LXR/RXR activation, glycosaminoglycan degradation and hypoxia signaling in the cardiovascular system, which includes hypoxia initiation factor 1α (HIF1α) signaling (data not shown). Moreover, Gene Set Enrichment Analysis (GSEA) confirmed the decreases found in inflammatory signaling pathways, such as eicosanoid metabolism, IL6 and MAP kinase signaling. Also oxidative stress, cell adhesion and PPAR signaling were down-regulated (data not shown).

Interestingly, pathway analysis of genes changed in the HOSF group also showed down-regulation of genes involved in inflammation and cell adhesion (**Figure 3**). However, the expression of fewer genes was changed upon HOSF intake, and consequently, fewer genes were observed in the changed pathways. In addition to the genes present in the analyzed pathways, several other genes involved in similar processes were down-regulated as well, and are depicted in **Figure 4**. This list shows various inflammatory genes, such as NF-κB target genes, oncostatin M (*OSM*), Ig-like receptors and interleukin receptors, that were down-regulated in the high EPA/DHA group. Also, gene expression of several plaque stability-related metallopeptidases was decreased after 26 weeks of EPA/DHA supplementation (**Figure 4**).

Process	Gene name	Description	Entrez ID	Mean FC EPADHA	EPADHA: q-value	MUFA: q-value
Inflammation						
inflammatory signaling						
	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1051	-1.11		
	DUSP1	dual specificity phosphatase 1	1843	-1.15		
	DUSP2	dual specificity phosphatase 2	1844	-1.50		
	MKNK1	MAP kinase interacting serine/threonine kinase 1	8569	-1.06		
	OSM	oncostatin M	5008	-1.24		
	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	3572	-1.20		
	IL13RA1	interleukin 13 receptor, alpha 1	3597	-1.17		
	NFIL3	nuclear factor, interleukin 3 regulated	4783	-1.25		
	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	2114	-1.16		
	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	1978	-1.12		
	H3F3B	H3 histone, family 3B (H3.3B)	3021	-1.09		
	LILRB3	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	11025	-1.11		
	LILRA2	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2	11027	-1.15		
	LILRA5	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	353514	-1.32		
	C5AR1	complement component 5a receptor 1	728	-1.19		
	FADD	Fas (TNFRSF6)-associated via death domain	8772	1.15		
NF-kB signaling						
	IRAK3	interleukin-1 receptor-associated kinase 3	11213	-1.32		
	IRAK1	interleukin-1 receptor-associated kinase 1	3654	-1.09		
	MAP3K3	mitogen-activated protein kinase kinase kinase 3	4215	-1.08		
	GSK3B	glycogen synthase kinase 3 beta	2932	-1.14		
	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	7133	-1.07		
	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	7128	-1.34		
	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	5567	1.08		
	PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	5287	1.08		
	LTA	lymphotoxin alpha (TNF superfamily, member 1)	4049	1.19		
eicosanoid synthesis						
	LTA4H	leukotriene A4 hydrolase	4048	-1.06		
	ALOX5	arachidonate 5-lipoxygenase	240	-1.12		
	GPX3*	glutathione peroxidase 3 (plasma)	2878	-1.26		
	TBXAS1	thromboxane A synthase 1 (platelet, cytochrome P450, family 5, subfamily A)	6916	-1.12		
	CBR3	carbonyl reductase 3	874	1.15		
Toll like receptor signaling						
	TLR8	toll-like receptor 8	51311	-1.11		
	TLR5	toll-like receptor 5	7100	-1.18		
	CD14	CD14 molecule	929	-1.12		
Cell adhesion						
integrin mediated cell adhesion						
	ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)	3684	-1.08		
	ITGA7	integrin, alpha 7	3679	-1.06		
	GRB2	growth factor receptor-bound protein 2	2885	-1.07		
	GAB2	GRB2-associated binding protein 2	9846	-1.20		
	VAV2	vav 2 guanine nucleotide exchange factor	7410	-1.07		
	VAV3	vav 3 guanine nucleotide exchange factor	10451	-1.09		
	PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	5058	-1.13		
	CD2	CD2 molecule	914	1.11		
chemokine signaling						
	CXCL16*	chemokine (C-X-C motif) ligand 16	58191	-1.17		
	CXCR4	chemokine (C-X-C motif) receptor 4	7852	-1.28		
	CCR3	chemokine (C-C motif) receptor 3	1232	-1.15		
	PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	23236	-1.19		
	PLCL2	phospholipase C-like 2	23228	-1.07		
	CXCL5	chemokine (C-X-C motif) ligand 5	6374	1.18		
	CX3CR1	chemokine (C-X3-C motif) receptor 1	1524	1.14		
	CCR5	chemokine (C-C motif) receptor 5	1234	1.24		
	PF4	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	5196	1.12		
	PF4V1	platelet factor 4 variant 1	5197	1.21		
	CXCR3	chemokine (C-X-C motif) receptor 3	2833	1.20		
Macrophage differentiation/foam cell formation						
scavenger receptor activation						
	CXCL16*	chemokine (C-X-C motif) ligand 16	58191	-1.17		
	CD36*	CD36 molecule (thrombospondin receptor)	948	-1.13		
	LDLR*	low density lipoprotein receptor (familial hypercholesterolemia)	3949	-1.18		
	CD163	CD163 molecule	9332	-1.12		
	SCARB2	scavenger receptor class B, member 2	950	-1.09		
adipogenesis						
	ADFP*	adipose differentiation-related protein	123	-1.01		
	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	6319	-1.12		
	NCOA1	nuclear receptor coactivator 1	8648	-1.12		
	RXRA	retinoid X receptor, alpha	6256	-1.12		
	NRIP1	nuclear receptor interacting protein 1	8204	-1.28		
	IRS2	insulin receptor substrate 2	8660	-1.30		
	GADD45B	growth arrest and DNA-damage-inducible, beta	4616	-1.12		
	DDIT3	DNA-damage-inducible transcript 3	1649	-1.13		
	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	4282	1.07		
plaque stability						
	MMP25	matrix metallopeptidase 25	64386	-1.07		
	MMP15	matrix metallopeptidase 15 (membrane-inserted)	4324	-1.07		
	TIMP2	TIMP metallopeptidase inhibitor 2	7077	-1.12		
	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	8754	-1.12		
	ADAMTSL4	ADAMTS-like 4	54507	-1.15		
	ADAM8	ADAM metallopeptidase domain 8	101	-1.05		
Oxidative stress						
ROS protection						
	SOD2	superoxide dismutase 2, mitochondrial	6648	-1.14		
	GPX3*	glutathione peroxidase 3 (plasma)	2878	-1.26		
	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	4094	-1.16		
	JUNB	jun B proto-oncogene	3726	-1.16		
	JUND	jun D proto-oncogene	3727	-1.06		
	FTH1	ferritin, heavy polypeptide 1	2495	-1.15		
	BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	571	-1.15		
HIF signaling						
	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3091	-1.10		
	VEGFA	vascular endothelial growth factor A	7422	-1.19		
	CREB5	cAMP responsive element binding protein 5	9586	-1.29		
	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	5728	-1.14		
	UBE2E2	ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	7325	-1.01		
	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	4792	-1.17		
	UBE2D1	ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast)	7321	-1.11		
nitrix oxide production						
	NOS3	nitric oxide synthase 3 (endothelial cell)	4846	1.10		
	NOSIP	nitric oxide synthase interacting protein	51070	1.07		

Process	Gene name	Description	Entrez ID	Mean FC EPADHA	EPADHA: q-value	MUFA: q-value
PPAR signaling						
	ACVR2A	activin A receptor, type IIA	92	-1.01		
	ADIPOR2	adiponectin receptor 2	79602	-1.06		
	CD36*	CD36 molecule (thrombospondin receptor)	948	-1.13		
	LDLR*	low density lipoprotein receptor (familial hypercholesterolemia)	3949	-1.18		
	ADFP*	adipose differentiation-related protein	123	-1.01		
Cell cycle						
	CDK2	cyclin-dependent kinase 2	1017	1.07		
	TP53	tumor protein p53 (Li-Fraumeni syndrome)	7157	1.07		
	CDK4	cyclin-dependent kinase 4	1019	1.08		
	PCNA	proliferating cell nuclear antigen	5111	1.09		
	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	3265	1.10		
	LCK	lymphocyte-specific protein tyrosine kinase	3932	1.11		
	MCM6	minichromosome maintenance complex component 6	4175	1.11		
	CCND2	cyclin D2	894	1.14		
	RPA2	replication protein A2, 32kDa	6118	1.06		
	RPA3	replication protein A3, 14kDa	6119	1.08		

down-regulated	
not significant	
p val <0.1 and >0.05	
p val <0.05 and >0.01	
p val < 0.01	
up-regulated	
not significant	
p val <0.1 and >0.05	
p val <0.05 and >0.01	
p val < 0.01	

Figure 4 Genes changed after EPA/DHA supplementation reflected in various functional groups that were overrepresented by the pathway analyses software programs GenMAPP, IPA and GSEA, supplemented with changed genes also related to these processes. Asterisks indicate that the genes appear in multiple functional groups. FC, fold change.

Additionally, we examined the expression changes of previously described PPARα target genes in PBMCs[89] and observed that 14 out of these 106 PPAR response element-containing PPARα target genes were down-regulated, and two were up-regulated (**Table 3**).

Table 3 Previously described PPARα target genes in PBMCs[89] found to be changed upon high EPA/DHA supplementation. FC, fold change; FDR Q, false discovery rate Q value.

Gene Description	Gene name	Entrez ID	Mean FC	FDR Q
Heparin-binding EGF-like growth factor	HBEGF	1839	-1.40	0.021
Pyruvate dehydrogenase kinase isozyme 4	PDK4	5166	-1.25	0.027
Zinc finger E-box-binding homeobox 2	ZEB2	9839	-1.21	0.004
Vascular endothelial growth factor A	VEGFA	7422	-1.19	0.024
Small inducible cytokine B16 precursor	CXCL16	58191	-1.17	0.010
Transcription regulator protein BACH1	BACH1	571	-1.15	0.003
Microphthalmia-associated transcr. factor	MITF	4286	-1.15	0.021
Myeloid-assoc. differentiation marker	MYADM	91663	-1.14	0.003
Platelet glycoprotein 4	CD36	948	-1.13	0.049
MARVEL domain containing 1	MARVELD	83742	-1.12	0.027
TNF receptor superfamily member 8	TNFRSF8	943	-1.11	0.045
Dihydropyrimidinase-related protein 2	DPYSL2	1808	-1.10	0.026
Adipose differentiation-related protein	ADFP	123	-1.10	0.031
Suppressor of tumorigenicity protein 14	ST14	6768	-1.09	0.037
Uncharacterized protein C15orf17	C15orf17	57184	1.11	0.043
Protein Chibby	CBY1	25776	1.11	0.036

To determine whether a low dose of 0.4 g EPA/DHA daily can induce similar gene expression changes as a high dose of 1.8 g EPA/DHA daily, a selection of six genes out of several changed pathways were analyzed with Q-PCR in the whole study population of 111 individuals (**Figure 5**). QPCR of *CD36*, *PDK4*, *LTA4H*, *ADFP, CD14*

and HIF1α revealed that consumption of 0.4 g EPA/DHA daily for 26 weeks also resulted in a down-regulation in expression of these genes and that the effects were intermediate between the effect of the 1.8 g EPA/DHA group and the HOSF group. These data also confirm the microarray results, as reflected by the same direction of gene expression changes.

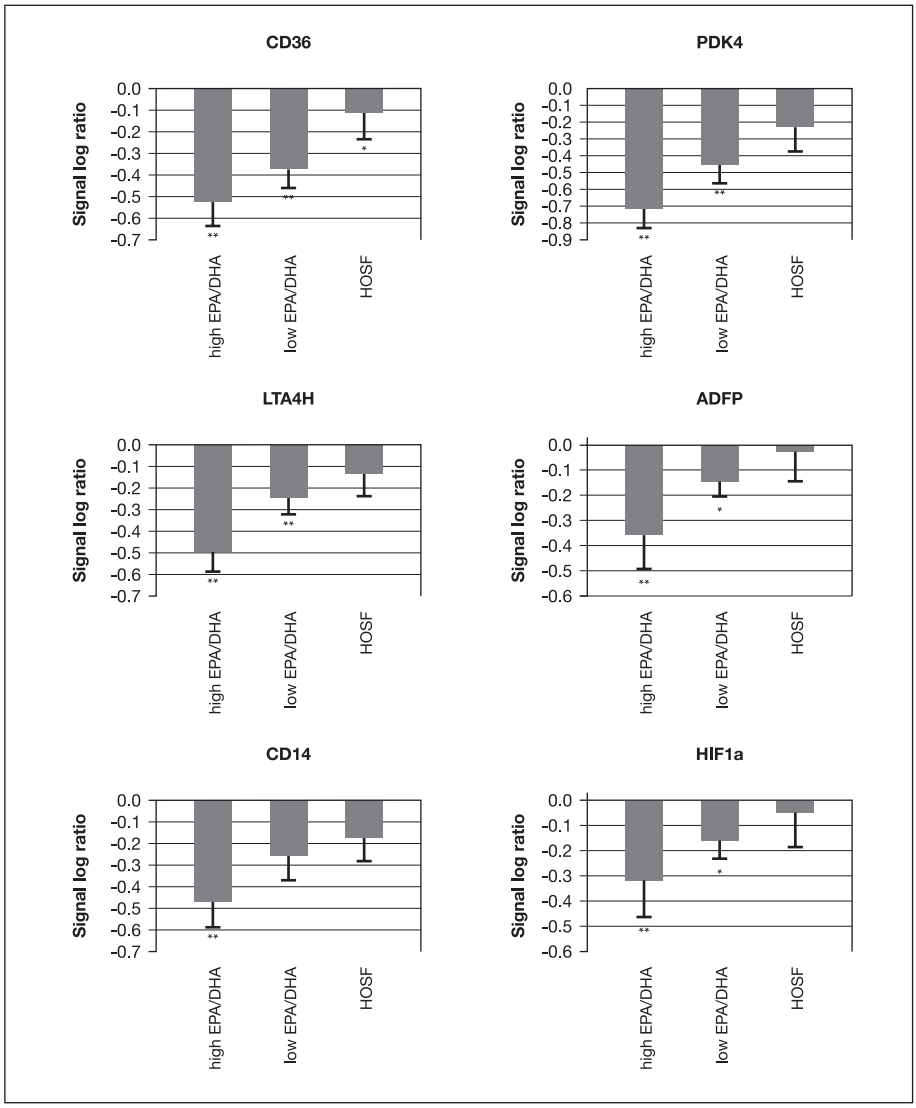


Figure 5 (left page) Q-PCR results of a selection of genes changed upon high EPA/DHA supplementation for the high EPA/DHA, low EPA/DHA and HOSF intervention groups. CD36, CD36 antigen; PDK4, pyruvate dehydragonase kinase 4; LTA4H, leukotriene A4 hydrolase; ADFP, adipose differentiation related protein; CD14, CD14 antigen; HIF1a, hypoxia induced factor 1 alpha. Values are mean ±SEM. ** $p<0.01$ and * $p<0.05$ compared with baseline.

Discussion

In this study we assessed changes in PBMC gene expression profiles after fish oil supplementation and a control in a 26-week double-blind and randomized intervention trial in an elderly population. Supplementation of 1.8 g of EPA/DHA daily, the equivalent of the consumption of ten portions of fatty fish weekly, results in anti- inflammatory- and anti-atherogenic-like PBMC gene expression profiles. In addition, supplementation with 0.4 g of EPA/DHA daily, representing two portions of fatty fish weekly, resulted in similar gene expression changes, but with a lower magnitude.

The effects of fish oil on health have been extensively studied. However, the impact of fish oil supplementation on whole genome gene expression profiles in PBMC has not been examined. To our knowledge, only one study examined the effect of fish oil supplementation on human leukocyte gene expression profiles[95]. However, in this study a macroarray containing only 588 genes, was examined in an RNA pool of eight volunteers and no control group was included. The anti-inflammatory-like gene expression profile observed in the present study is mainly characterized by decreased gene expression of inflammatory genes, including several NF-κB target genes, pro-inflammatory cytokines, chemokines and eicosanoid synthesis. The above described study also observed a decrease in expression of some cytokine, chemokine receptor and stress response genes. In addition, several other intervention studies with varying doses of n-3 fatty acids such as α-linolenic acid (ALA) or EPA/DHA/fish oil, observed a decrease in plasma inflammatory markers such as CRP, E-selectin and TNFα[96-98] or a reduction in cytokine formation after *ex vivo* stimulation in mononuclear cells[99,100]. The decrease in eicosanoid formation we found was characterized by a down-regulation in the gene expression of enzymes involved in eicosanoid synthesis, such as LTA4H and ALOX-5. It is known that n-3 PUFA supplementation can result in a decrease in pro-inflammatory arachidonic acid derived eicosanoids[28]. For example, various studies show that supplementation of fish oil or DHA for 4 to

17 weeks resulted in a decrease in the *ex vivo* stimulation-induced production of eicosanoids, such as prostaglandin E2, thromboxane B2, leukotriene B4 and leukotriene E4 (LTE4) in inflammatory cells of healthy volunteers[28]. In addition, intake of n-3 fatty acids for 6 weeks in 14 human volunteers reduced urinary excretion of LTE4 (101). Moreover, a 3-week n-3 enriched diet intervention study in mice suppressed the generation of both leukotrienes and prostaglandins during acute inflammation[102]. Hence, our observations on PBMC gene expression nicely reflect n-3 PUFA-induced effects on plasma and urinary inflammatory markers and *ex vivo* stimulated inflammatory responses.
The group of subjects that consumed the HOSF capsules showed a similar, albeit less pronounced effect on some of the inflammatory pathways. The overlapping genes were almost all changed in the same direction, suggesting that some of the effects on inflammation, although clearly less apparent, are comparable with the EPA/DHA intervention. Beneficial effects of MUFAs on inflammation have been reported, but only limited data is available[103].

The anti-atherogenic gene expression profile that we observed in this study after EPA/DHA intake is characterized by a reduced expression of genes that play a role in several processes known to be involved in atherosclerotic plaque formation. The PBMC subpopulations exist of monocytes/macrophages, and T- and B-lymphocytes, which can play a role in adhesion, infiltration and subsequent foam cell formation during atherogenic development[104]. In our study we found an EPA/DHA induced decrease in integrin-mediated cell adhesion, characterized by down-regulation of genes encoding proteins such as integrin αM, a monocyte cell adhesion molecule[105], and growth factor receptor-bound protein 2. An n-3 PUFA-induced decrease in expression of monocyte/macrophage adhesion molecules has previously been reported, but only in animal and *in vitro* studies[106, 107]. Because leukocyte adhesion molecules promote binding of monocytes and T cells to the endothelium[104], the observed EPA/DHA-induced reduction in gene expression might lead to decreased rolling and adhesion of mononuclear leukocytes.
Besides an EPA/DHA induced decrease in cell adhesion, we also observed down-regulation in expression of several chemokine signaling genes, such as chemokine receptor *CXCR4*, which mediates migration of resting leukocytes[108]. However, we observed both up- and down-regulation of several adhesion molecules and chemokines related to these processes. Moreover, the increases

found in chemokines such as *platelet factor 4* (*PF4*), and its receptor, *CXCR3* are associated with increased risk of atherosclerosis[109], but on the other hand can inhibit leukocyte chemotaxis[110], and angiogenesis[111]. Additionally, an increase in the chemokine receptor CCR5 was found, which is known to have a higher expression in foam cells and activated T cells in mouse and human atheromas[112], but which is also known to clear chemokines from the site of activation[113].

Our study also revealed that EPA/DHA supplementation for half a year reduces scavenger receptor activity, as characterized by a lower expression of genes encoding CD36 and the LDL receptor. Moreover, we observed a decreased in adipogenesis, portrayed by a decrease in expression of genes involved in lipid accumulation, such as adipose differentiation-related protein (ADFP). Scavenger receptors, such as CD36 and LDLR, are involved in the uptake of particles such as oxidized low density lipoproteins (oxLDL). It has been shown that n-3 PUFA incubation in monocytes *in vitro* can reduce scavenger receptor mRNA and protein expression[114]. After uptake, the oxLDL can accumulate in intracellular droplets, which requires the action of lipid droplet proteins, such as ADFP[115]. Incubation of macrophages with PUFA-enriched chylomicron remnant-like particles resulted in a reduced lipid accumulation in these cells[116]. Similarly, studies in rats showed that EPA feeding inhibits cholesterol ester accumulation in macrophages in a dose-dependent manner[117]. The reduction in gene expression of scavenger receptors and ADFP upon EPA/DHA supplementation that we observe in human PBMCs are in line with these *in vitro* data and might imply that the monocytes present within the PBMCs are less prone to differentiate into foam cells after 26 weeks of EPA/DHA supplementation.

Besides the processes discussed above, we also observed changes in genes related to plaque stability, a process that normally occurs after infiltration and foam cell formation. Expression of genes of matrix metalloproteinases (MMPs) and disintegrin metalloproteinases (ADAMs), endopeptidases that can degrade the extracellular matrix, was down-regulated upon EPA/DHA intake. These genes are known to destabilize atherosclerotic plaques and thus can result in plaque rupture[118]. Also, *hypoxia inducible factor 1α* (*HIF1α*) and its main target gene *vascular endothelial growth factor* (*VEGF*) were down-regulated[119]. Both genes are known to regulate atherosclerotic plaque angiogenesis, which destabilizes and progresses the lesion. Plaque stability has been found to be increased after n-3 PUFA supplementation[120].

We also found various markers for oxidative stress, such as *superoxide dismutase 2, glutathione peroxidase 3* and *ferritin*, down-regulated after EPA/DHA intake. Oxidative stress is involved in the initiation and progression of atherosclerosis[121] and the observed reduction in anti-oxidants in this study may imply a decrease in oxidative stress in the leukocytes after intake of n-3 PUFAs. PUFAs have been shown to moderate oxidative stress, as measured in urine or blood in several[121, 122] but not in all studies[123]. Because highly unsaturated fatty acids are more prone to oxidation, concern exists that high intake of n-3 PUFA might lead to an increased susceptibility to oxidative stress. However, it has been suggested that in individuals consuming n-3 PUFAs, fatty acids are initially more readily oxidized, but that the total oxidation is lower[123]. Long term intake of EPA/DHA as assessed in this study might therefore result in reduced oxidative stress.
High EPA/DHA intake in our study also increased expression of nitric oxide synthase 3 (NOS3/eNOS), which plays a role in protection of the vessel wall from atherosclerosis[124]. Increased expression of eNOS in murine macrophages and *in vitro* in human vascular wall cells after supplementation or incubation with EPA/DHA has been shown before[125-127]. Importantly, the studies described, all illustrate short term *in vitro* experiments or animal studies, while we observed our effects in humans after 26 weeks of n-3 PUFA intake.

In this study, PBMC gene expression changes nicely reflect anti-inflammatory and anti-atherogenic changes and we may speculate that EPA/DHA supplementation will improve the pre-atherosclerotic condition in elderly people. Another interesting point to discuss is whether the changes in gene transcription profiles are due to a direct effect of EPA/DHA in mononuclear cells or whether the changes reflect the response of PBMCs to EPA/DHA induced systemic adaptations. This is nicely illustrated by the unexpected finding of down-regulation of PPARα target genes after EPA/DHA supplementation. Based on mice and *in vitro* studies, in which a more pronounced expression of PPAR target genes upon EPA and DHA intake than upon saturated or monounsaturated fatty acid intake was shown[90], we expected an increase in PPARα target genes upon EPA/DHA supplementation instead of the observed decreases. A possible explanation for these unexpected findings may lie in systemic adaptations by effects of EPA/DHA on gut or liver, resulting in reduced plasma FFA and TG concentration. The observed down-regulation of PPAR target genes, but also of other genes such as scavenger

receptors, might have been caused by these reduced plasma FFA and TG concentrations. The down-regulation of these genes might be due to systemic action of EPA/DHA as a consequence of long term adaptation, rather than by a direct effect in the PBMCs. Hence, PBMC gene expression profiles could also be viewed as a reflection of the physiological state of subjects, as we have shown previously[67]. The potential of gene expression profiles as biomarkers has already been recognized in the pathological field[8, 86], but this study demonstrates that also nutritional interventions can significantly induce changes in PBMC gene expression profiles.

One of the remarks that should be made concerning the nutritional intervention is that the high dose of EPA/DHA in this study is comparable to a relatively high consumption of ten portions of fatty fish weekly. However, Q-PCR results show that a lower dose, representing the intake of two portions of fatty fish weekly, resulted in similar, but lower, gene expression changes. This suggests that lower amounts of fish intake may have similar effects on PBMC gene expression profiles, especially when taken for a longer period. The HOSF group showed an even smaller, but still detectable modulatory effect on expression of inflammatory genes. The HOSF capsules contained approximately four grams of oleic acid, which is an addition of about 16% MUFA for woman and 13% MUFA for men over 65 years old to the normal Dutch, western diet[128]. The small changes seen in the HOSF group might have been the result of this increased intake of MUFA.

Our study population consisted of elderly individuals, which likely express a slightly more pro-inflammatory and pro-atherosclerotic gene expression profile, since it has been shown that ageing is associated with chronic, low grade increased inflammatory activity[129]. This makes the studied population suitable to detect nutritional effects on these transcriptional profiles. The observed effects of EPA/DHA on PBMC gene expression profiles can therefore not directly be translated to younger populations. Interestingly, a recent study in obese men showed that weight loss reduced expression of inflammatory genes in PBMCs[87]. In the present study we find similar anti-inflammatory changes in PBMC gene expression of lean subjects, merely by supplementation with 1.8 g/d, and even 0.4 g/d of EPA/DHA. This emphasizes the potential of PBMC gene expression profiling to determine the effects of nutrition on human health status.
In conclusion, this study reveals that intake of the n-3 PUFAs EPA and DHA for 26

weeks alters gene expression profiles of PBMCs in the direction of a more anti-inflammatory and anti-atherogenic-like profile. Moreover, this nutrigenomics study demonstrates that PBMC profiling can not only reflect pathological conditions or the physiological state, but can also reflect metabolic changes due to long term nutritional adaptations.

Acknowledgments

We thank Jenny Jansen and Shohreh Keshtkar for excellent technical assistance. This work was supported by the Dutch Dairy Association, Zoetermeer, The Netherlands, the Nutrigenomics consortium of TI Food and Nutrition, Wageningen, The Netherlands, the European Nutrigenomics Organisation (NuGO) and the Netherlands organization for health research and development (ZonMw, grant number 6100.0004), The Hague, the Netherlands.

Author contributions

LdG and JMG designed the trial; OvdR performed the trial; LAA designed the research; MB and MGB performed the research; MB and ND analyzed data; MB wrote the paper; and LAA and MM critically reviewed the paper.

The authors declare no conflict of interest.

Chapter 5

Human peripheral blood cells show distinct fatty acid-specific postprandial gene expression profiles

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Submitted for publication
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Abstract

Background

Dietary polyunsaturated fatty acids (PUFAs) have been suggested to have a variety of beneficial effects, of which the effects on inflammation and cardiovascular diseases are most generally accepted. Immune cells play an important role in these processes, but the mechanisms of action of PUFAs on these cells are still not completely understood. It is known that PUFAs can elicit effects on gene expression. Since most individuals are in a postprandial state for the main part of the day, knowledge about direct effects of fatty acids on immune cells is highly valuable.

Design

In a single blind cross over study, 21 healthy male volunteers consumed shakes enriched in PUFAs, monounsaturated (MUFA) or saturated fatty acids (SFA) in random order. Before and at several time points after intake of the shakes blood was drawn and PBMCs were isolated to use for gene expression analysis. Whole genome gene expression profiles were examined before and 6 hours after intake of the PUFA and SFA shakes. Additionally, *ex vivo* incubations of human PBMC with different fatty acids were performed.

Results

Whole genome expression analysis showed distinct differences between PUFA and SFA consumption in PBMCs. Consumption of the PUFA shake resulted in a decrease expression of genes in LXR signaling, while SFA intake showed an increase in these same genes. PUFA intake also demonstrated an increase in genes related to cellular stress responses. MUFA intake had an intermediate effect on several genes. Additional *ex vivo* experiments revealed a direct effect of DHA on PBMC gene expression.

Conclusions

PBMC microarray analysis allows to detect subtle differences in the effects of different types of dietary fatty acids on whole genome gene expression profiles.

Introduction

Dietary polyunsaturated fatty acids (PUFAs) are suggested to have a variety of beneficial effects, of which the effects on inflammation (28) and cardiovascular diseases[130] are the most generally accepted. Immune cells play an important role in inflammation and the development of cardiovascular disease. However, the mechanisms of action of PUFAs on the functions of immune cells are still not completely understood[131]. It is known that PUFAs can mediate their effects through changes in gene expression, mainly regulated via activation of transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and LXR[59,132]. Whole genome transcriptional profiling of immune cells would elucidate all processes that PUFAs regulate on gene expression level. Most studies that examined the effect of PUFAs on human immune cells have been performed in long term intervention trials[133], and were related to the immunological function of these cells. However, whole genome expression studies have not been carried out. Only one study showed that intake of PUFA-rich fish oil for 2 months changed expression of several genes related to inflammation in leukocytes[95], but only a selected set of 588 genes were examined. Furthermore, measures in long term studies are mostly performed in fasted samples. However, most individuals are in a postprandial state for the greater part of every 24 hour period, and therefore knowledge about postprandial effects of different fatty acid types on these cells are highly valuable. It is unknown whether different fatty acids can elicit differential postprandial effects on gene expression of immune cells of human individuals, and what these effect will encompass. Therefore, we examined the postprandial effects of consumption of a shake containing 70 energy% of fat in the form of PUFA (65% of total fat), SFA (70% of total fat) or monounsaturated fatty acids (MUFA, 80% of total fat) on gene expression changes in PBMCs of 21 healthy men in a cross-over design. Whole genome microarray analyses were performed before and 6 hours after PUFA- and SFA-enriched shake consumption. To explore the response kinetics, Q-PCR analysis were performed before and at 5 time points after intake of all shakes.

Subjects and Methods

Recruitment of subjects

Twenty-one healthy male Caucasian volunteers, between 19 and 27 years of age were recruited from the Wageningen student population. Exclusion criteria were a body mass index (BMI) < 18 or > 27 kg/m^2, urine protein or glucose concentrations outside normal ranges or fasting blood glucose outside the normal range. Other exclusion criteria were: tobacco smoking, taking regular prescribed medication, received inoculations within 2 months of starting the study or planned to during the study, donated or intended to donate blood within 8 weeks of the first and last study samples, diagnosed with any long-term medical condition (e.g. diabetes, hemophilia, cardiovascular disease, anemia, gastrointestinal disease), experiencing symptoms of allergy, or vegetarian. Subjects were informed about the design and purpose of the study and provided full informed written consent.

Study design

At a screening visit, urine and fasting venous blood samples were taken to determine glucose concentrations in both urine and blood, and protein concentrations in urine.

In a cross-over design, the twenty-one male subjects randomly consumed three shakes enriched with polyunsaturated (PUFA), monounsaturated (MUFA) or saturated fatty acids (SFA) on three different days, with at least one week between each study day. Before each study day, subjects received identical meals containing less than 10 gram of fat, which they had to consume before 8 pm the previous evening. On the morning of each study day the subjects came to the University in fasted condition and before the start of the study a canula was place in their forearm. At the start of the study 6 ml of blood was drawn into EDTA tubes for plasma isolation and 24 ml into BD Vacutainer Cell Preparation Tubes with sodium citrate (BD, Breda, The Netherlands) for PBMC isolation. Directly after the first blood sampling the subjects were given a shake, which they had to consume within 10 minutes. Subsequently, every 2 hours blood was drawn, until 8 hours after intake of the shake. During a study day, the subjects were not allowed to eat or drink anything except water, which they were advised to drink regularly (approximately 150 ml each hour). After 8 hours, the volunteers received a meal. Subjects were asked to keep record of their physical condition

during the complete study period, which lasted from January 14th, 2008 till March 7th, 2008. The study protocol was approved by the Medical Ethical Committee of Wageningen University.

Shake constituents

The shakes contained 350 ml of low-fat yoghurt, 100 ml of skimmed milk, 7,5 gram sugar and 55 gram of the test fat. Vitamin E (165 mg) was added as antioxidant. The test fats were 65% polyunsaturated fatty acids, of which approximately 40% docosahexaenoic acid (DHA) (PUFA shake, Marinol D-40, Lipid Nutrition, Wormerveer, the Netherlands); 80% monounsaturated fatty acids, predominantly oleic acid (MUFA shake, high oleic acid sunflower oil, Aldoc BV, Schiedam, the Netherlands), or 70% saturated fatty acids (SFA shake, butter). Composition of the shakes was calculated using the computer package KOMEET[134]. This program is based on the database of the Dutch Nutrient Databank[135]. Fat percentages were measured by gas–liquid chromatography (GLC)[136].

Blood glucose and plasma free fatty acids and triglycerides

Blood glucose concentrations were determined with Accu-Chek Compact blood glucose meters (Roche Applied Science). Immediately after blood drawing, blood in the EDTA tubes was centrifuged (750 x *g*, 4 °C, 10 min), and plasma was stored at -80˚ C. Plasma free fatty acids and triglycerides were measured by gas–liquid chromatography (GLC).

PBMC isolation and flow cytometry analysis

Immediately after blood collection, PBMCs were isolated using the BD Vacutainer Cell Preparation Tubes according to the manufacturer's manual. Cells were counted at baseline and 6 hours after intake of the shakes, and tested for viability by trypan blue exclusion. Flow cytometry analysis was performed on 1.5 x 10^6 cells using a FACS Canto II flow cytometer (BD Biosciences). Data was analyzed using BD FACSDiva software (BD Biosciences).

Total RNA isolation

PBMC RNA was isolated from all PBMC samples using the Qiagen RNeasy Micro kit (Qiagen, Venlo, the Netherlands). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (Nanodrop technologies, Wilmington, Delaware

USA) and integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, UK).

Microarray processing

PBMC samples from baseline and 6 hours after intake of the PUFA and the SFA shakes were used for microarray analysis, resulting in a total of 84 arrays. Total RNA from PBMCs from all subjects was labeled using a one-cycle cDNA labeling kit (MessageAmpTM II-Biotin Enhanced Kit, Ambion, Inc.) and hybridized to human whole genome NuGO GeneChip arrays encoding 17,699 genes, designed by the European Nutrigenomics Organisation (NuGO) and manufactured by Affymetrix (Affymetrix Inc, Santa Clara, CA). Sample labeling, hybridization to chips and image scanning was performed according to the manufacturer's GeneChip Expression Analysis Technical Manual (Affymetrix).

Microarray analysis

All microarrays fulfilled our quality control criteria. Microarrays were analyzed using the reorganized oligonucleotide probes as described by Dai *et al* (2005)[68]. Dai *et al.* combined all individual probes for a gene, enabling the possibility to detect the overall transcription activity of a gene, based on the latest genome and transcriptome information, instead of the Affymetrix probe set annotation. Expression values were calculated using the Robust Multichip Average (RMA) method. RMA signal value estimates are based on a robust average of background corrected perfect match intensities and normalization was performed using quantile normalization[41, 42]. Only genes with normalized signals higher then 20 on at least 10 arrays were defined as 'expressed' and selected for further analysis. Genes were defined as 'changed' when comparison of the normalized signal intensities showed an FDR Q-value[93] lower than 0.05 in a two-tailed paired *t* test with Bayesian correction (Limma)[69].

Pathway analysis

Pathway analysis was performed using Ingenuity Pathway Analysis 5.5 (Ingenuity Systems). Array data has been submitted to the Gene Expression Omnibus, accession number GSE13466.

cDNA synthesis and real time PCR

RNA from all subjects and shakes was reverse transcribed with the use of a cDNA synthesis kit (Promega, Leiden, the Netherlands). Standard quantitative real-time polymerase chain reaction (Q-PCR) was performed with the use of Platinum Taq DNA polymerase and SYBR Green on an iCycler PCR machine (Bio-Rad Laboratories BV) and duplicated at least twice. Primer sequences used in the PCRs were chosen based on the sequences available in PRIMERBANK (http://pga.mgh.harvard.edu/ primerbank/index.html). Q-PCR data were normalized by measuring cycle threshold ratios between candidate genes and a housekeeping gene, human ribosomal protein LP0, which was shown to be consistent within PBMCs[45].

PBMC incubation studies

Blood from 4 healthy Caucasian male donors, aged between 26 and 42 yr, was obtained from the blood bank (Sanquin, Nijmegen) and PBMCs were isolated directly after arrival using Ficol-paque Plus density gradient centrifugation (Amersham Biosciences, Roosendaal, the Netherlands). PBMCs were incubated in RPMI1640 medium with 2 mmol/L L-glutamine, 10% fetal bovine serum and antibiotics (penicillin and streptomycin) in the presence of 5% CO_2 at 37°C. at 1.0 x 10^6 cells per ml, for 12 hours. Bovine serum albumin was added to bind fatty acids and the following fatty acids were tested; palmitic acid (C16.0, 200 µmol/L), oleic acid (C18.1, 500 µmol/L) and docosahexaenoic acid (C22.6, 10 µmol/L). Concentrations were based on physiologically normal levels of the different fatty acid types found in plasma of volunteers in a previous study[67]. These were compared to KOH solution, in which the fatty acids were dissolved, as control. All donors gave full written informed consent.

Statistical methods

In this cross-over study, each subject consumed the three shakes and is used as his own reference. Data are given as mean ± SEM, n=21. Incremental shake responses (means over 21 determinations) are expressed as variations in concentration or relative gene expression value over baseline (fasting baseline values being zero). The 0 to 8 hour incremental area under the curve (AUC) was calculated by the trapezoidal method. The significance of the difference among the experimental shakes, the 0 to 8 hour time points for a given shake and the difference between shake AUCs were assessed using a repeated measures ANOVA

and a Least Significance Difference (LSD) post hoc test. A two tailed paired t-test was used to determine significant differences in percentages of blood cells in PBMCs between baseline and 6 hours after intake of the shakes. Statistical significance was accepted at $p<0.05$. All calculations were performed by using the SPSS-software package 15.0.1 (Chicago, Illinois).

Results

Subject characteristics

Twenty-one healthy men were included in the study, who returned to the University on three separate study days to consume a shake enriched with polyunsaturated (PUFA), monounsaturated (MUFA) or saturated fatty acids (SFA). Microarray analyses were performed for the PUFA and SFA enriched shakes. Subject characteristics, as measured during the screening visit, are shown in **Table 1**. The shakes contained 2824 kJ of energy and consisted of 18.4 grams protein, 34 gram of carbohydrates and 55 gram of fat. The fatty acid composition was determined by GLC and is shown in **Table 2**. No differences were observed in blood cell subpopulations between the study days and no changes were found after intake of the PUFA or SFA shakes, although the latter was tested in five individuals, only (data not shown).

Table 1 Subject characteristics of the men participating in the study

	Mean (n=21)	Range
Age (y)	21.5	18 - 27
Height (m)	1.84	1.76 - 1.96
Weight (kg)	74.4	62 - 87
BMI (kg/m^2)	22.1	18.1 - 26.3
Fasting glucose (mmol/L)	4.8	4.3 - 5.5

Table 2 Percentages of fatty acids as measured by gas–liquid chromatography[136]

	PUFA	SFA	MUFA
Total fat (En%)	71.5	71.5	71.5
Saturated (% of total fat)	18.51	70.73	7.63
Monounsaturated (% of total fat)	15.04	25.46	84.62
Polyunsaturated (% of total fat)	65.77	2.39	7.75
n-6 polyunsaturated (% of total fat)	6.77	1.35	7.75
n-3 polyunsaturated (% of total fat)	59.00	1.04	0.00
Docosahexaenoic acid (% of total fat)	48.54	0.00	0.00
Eicosapentaenoic acid (% of total fat)	7.24	0.00	0.00

Plasma FFA and TG, and blood glucose concentrations changed in time

Intake of all shakes resulted in a similar decrease in blood glucose and increase in total FFA in time. All shakes increase plasma TG concentrations, but the PUFA shake resulted in significantly higher TG concentration at t=6 and at t=8 hours compared to the SFA shake at the same time points. The MUFA shake appeared to have an intermediate effect. No significant differences in total response, as measured by incremental area under the curves (AUC), of triglycerides, glucose and FFA responses were observed between the shakes (**Figure 1).**

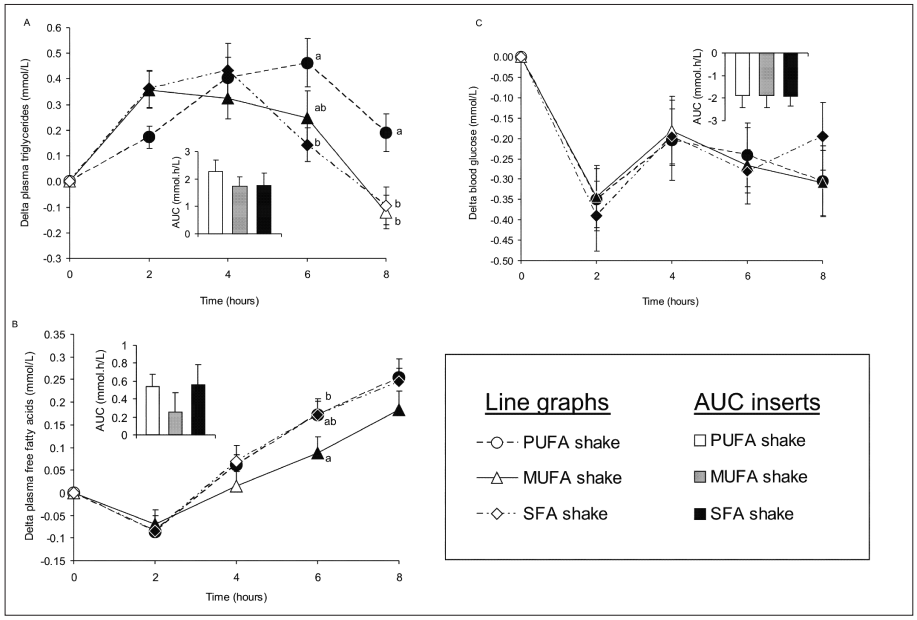

Figure 1 Effects of consumption of three shakes, providing polyunsaturated (PUFA), monounsaturated (MUFA) or saturated fatty acids (SFA), on plasma triglycerides (A), plasma total free fatty acids (B), and blood glucose (C) in healthy men. Values are means ± SEM, n = 21. The incremental areas under the curve (AUC) are shown in inserts. Different letters indicate a difference (p <0.05) between shakes at a given time. For a given test shake, a filled symbol indicates that the corresponding value differs (p <0.05) from baseline (0 h) value.

Microarray analysis

Microarray hybridization was performed on PBMC RNA that was collected at baseline and 6 hours after consumption of the PUFA shake and the SFA shake of all 21 subjects. All arrays passed the quality criteria. Changes in gene expression

were determined by comparison of the microarray results of the samples of t=6 hours with t=0 hours within all individuals in both intervention groups. From the 17,699 genes present on the microarray, 13,365 genes were defined as expressed in PBMCs (**Figure 2**). Consumption of the PUFA shake resulted in expression changes of 437 genes, whereas consumption of the SFA shake resulted in expression changes of 297 genes (**Figure 2**). Of these genes, 146 genes overlapped between the groups, of which all except two were changed in the same direction. These two genes, *ABCG1* and *SREBF1*, were down-regulated after PUFA shake intake, and up-regulated after SFA shake intake. Beside determining which genes were significantly changed within the shakes, we also elucidated whether gene expression was significantly changed between the shakes. A strict FDR Q-value below 0.05 revealed changes in expression between shakes for 3 genes, *ABCG1*, *SREBF1* and *ABCA1*.

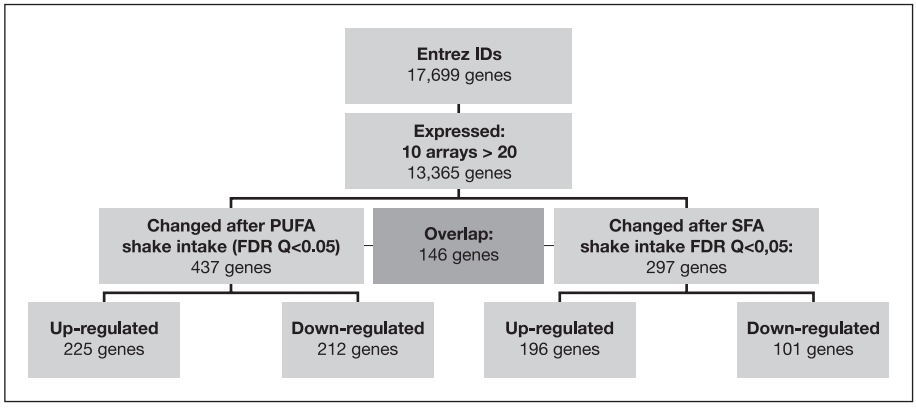

Figure 2 Gene selection procedure and criteria FDR Q, false discovery rate Q value.

Gene expression changes

To elucidate the role of the various genes which expression was changed after consumption of the shakes, pathway analysis was performed. This revealed that those genes were involved in processes related to Liver X receptor (LXR) signaling, oxidative stress, inflammation, carbohydrate metabolism, and a variety of other processes (**Figure 3**).

Figure 3 (right page) Pathways changed in PBMC gene expression profiles after consumption of the PUFA or SFA shakes, grouped by processes. Pathway analysis was performed with the program Ingenuity Pathway Analysis.

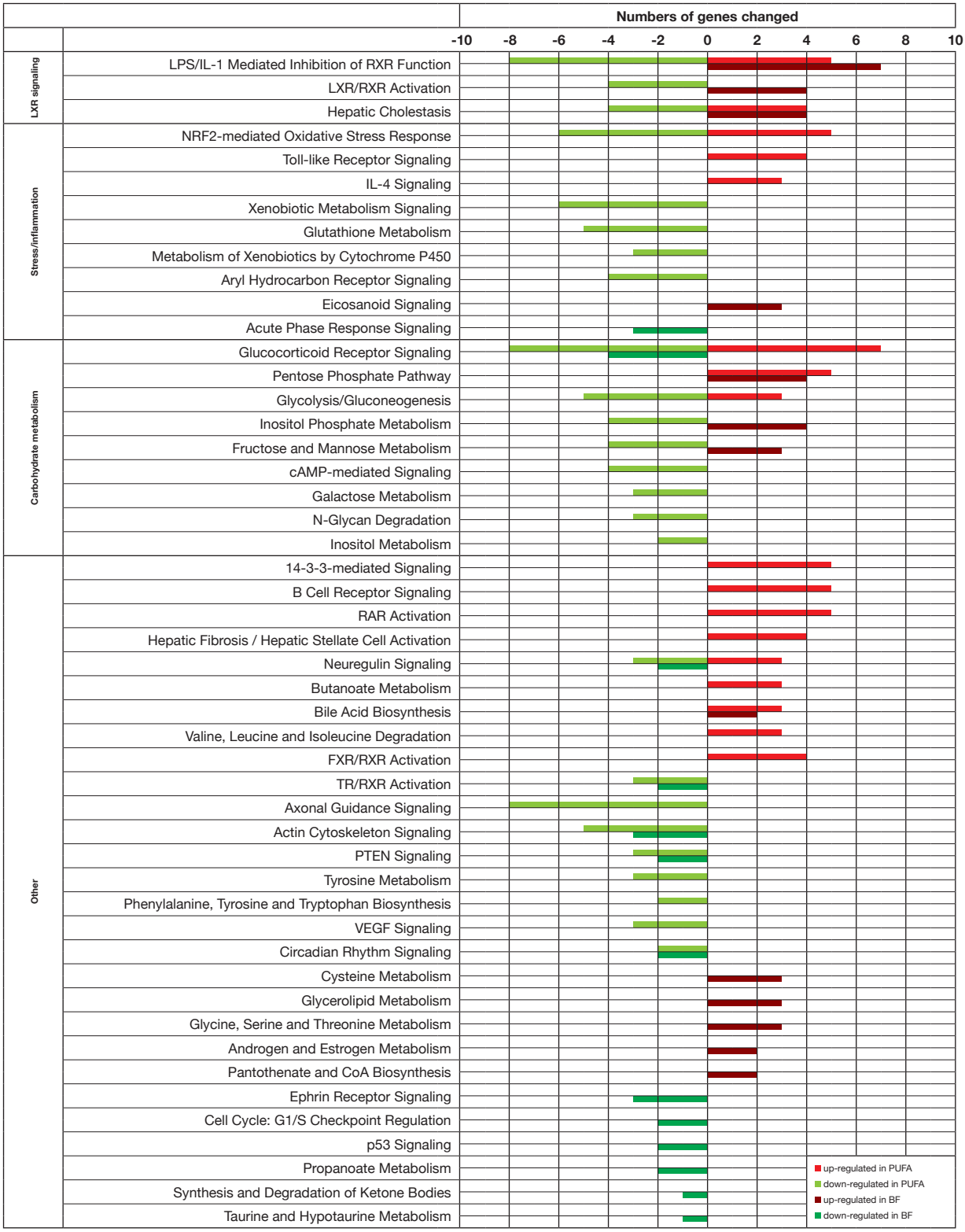
Numbers of genes changed
-10
-8
-6
-4
-2
0
2
4
6
8
10
LXR signaling
Stress/inflammation
Carbohydrate metabolism
Other
LPS/IL-1 Mediated Inhibition of RXR Function
LXR/RXR Activation
Hepatic Cholestasis
NRF2-mediated Oxidative Stress Response
Toll-like Receptor Signaling
IL-4 Signaling
Xenobiotic Metabolism Signaling
Glutathione Metabolism
Metabolism of Xenobiotics by Cytochrome P450
Aryl Hydrocarbon Receptor Signaling
Eicosanoid Signaling
Acute Phase Response Signaling
Glucocorticoid Receptor Signaling
Pentose Phosphate Pathway
Glycolysis/Gluconeogenesis
Inositol Phosphate Metabolism
Fructose and Mannose Metabolism
cAMP-mediated Signaling
Galactose Metabolism
N-Glycan Degradation
Inositol Metabolism
14-3-3-mediated Signaling
B Cell Receptor Signaling
RAR Activation
Hepatic Fibrosis / Hepatic Stellate Cell Activation
Neuregulin Signaling
Butanoate Metabolism
Bile Acid Biosynthesis
Valine, Leucine and Isoleucine Degradation
FXR/RXR Activation
TR/RXR Activation
Axonal Guidance Signaling
Actin Cytoskeleton Signaling
PTEN Signaling
Tyrosine Metabolism
Phenylalanine, Tyrosine and Tryptophan Biosynthesis
VEGF Signaling
Circadian Rhythm Signaling
Cysteine Metabolism
Glycerolipid Metabolism
Glycine, Serine and Threonine Metabolism
Androgen and Estrogen Metabolism
Pantothenate and CoA Biosynthesis
Ephrin Receptor Signaling
Cell Cycle: G1/S Checkpoint Regulation
p53 Signaling
Propanoate Metabolism
Synthesis and Degradation of Ketone Bodies
Taurine and Hypotaurine Metabolism
up-regulated in PUFA
down-regulated in PUFA
up-regulated in BF
down-regulated in BF

Figure 4 shows a heat map of the genes involved in LXR signaling, of which expression was changed upon intake of at least one of the shakes. In most individuals SFA shake consumption resulted in an increase in expression of genes involved in LXR signaling such as *LXR* (*NR1H3*), *ABCG1*, *SREBF1* and *ABCA1*, whereas PUFA shake consumption resulted in a down-regulation of the same genes. PUFA shake consumption resulted in gene expression changes indicating both an increase and a decrease in oxidative stress (**Figure 3**). Glutathione metabolism genes (e.g. *GSTK1*, *GSTP1*, *MGST3*) were responsible for the down-regulation of this pathway and genes involved in activation of inflammatory and cellular stress pathways were responsible for the up-regulation of this pathway after PUFA shake consumption. SFA shake consumption did not show a strong effect in stress responses on pathway level (**Figure 3**). However, a variety of pro-inflammatory and stress-related genes were also up-regulated after SFA shake intake, including *MAP3K1, CD40* and *ICAM1* (data not shown). In addition, we examined the changes in expression of previously described PPARα target genes in human PBMCs[89]. We found that out of the total of 106 PPAR response element-containing PPARα target genes, 6 genes were changed after PUFA shake intake and 5 genes after SFA shake intake, of which 4 were overlapping between shakes, including *PDK4* (data not shown).

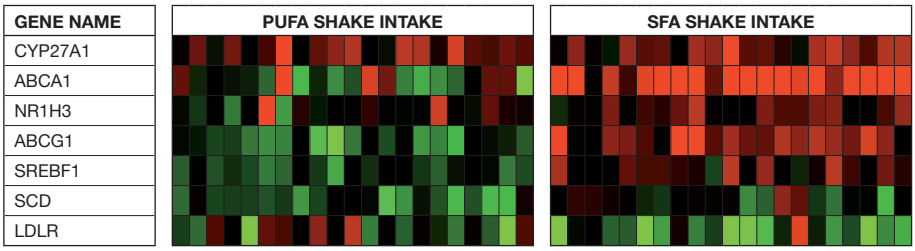

Figure 4 Heat map of genes changed after consumption of the PUFA or SFA shakes, within the process LXR signaling, with a FDR Q value below 0.05, in all 21 individuals. Red indicates up-regulation, green indicates down-regulation, black indicates no change.

To elucidate the response in gene expression in time and upon the MUFA shake, quantitative real time-PCR (Q-PCR) was performed for all time points after intake of the three shakes on a selection of genes. Genes were selected out of the pathways changed upon shake intake and included *PDK4, LXR, ABCA1, SREBF1, cJUN* and *GSTP1*. These genes showed that consumption of the shakes enriched

with PUFA, MUFA and SFA resulted in different gene expression responses in time, and also confirmed the microarray results, as reflected by the same direction of gene expression changes six hours after PUFA and SFA shake intake (**Figure 5**).

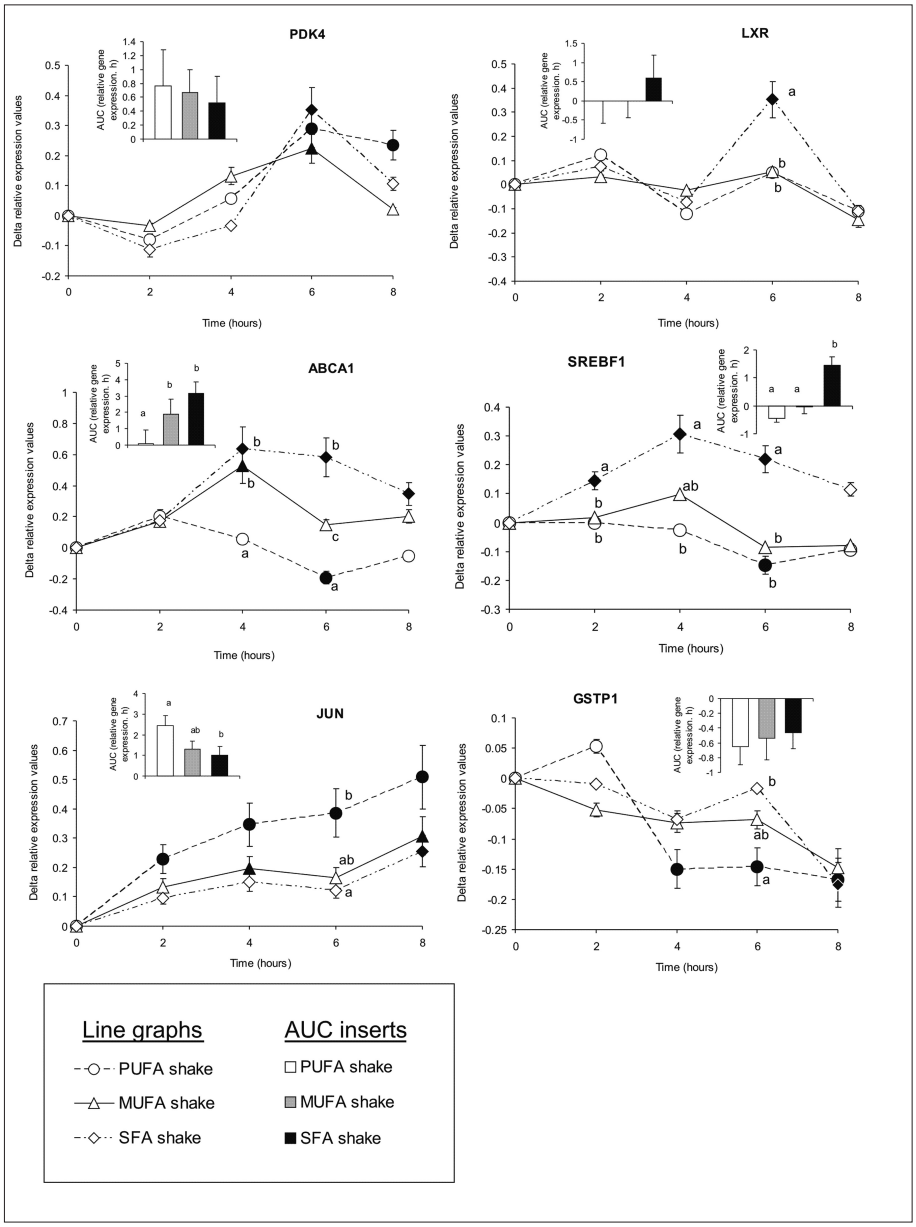

Figure 5 (previous page) Effects of consumption of three shakes, providing polyunsaturated (PUFA), monounsaturated (MUFA) or saturated fatty acids (SFA), on gene expression of several genes that were selected because they were changed 6 hours after intake of the PUFA and/or the SFA shake as assessed with microarray analysis. PDK4, pyruvate dehydrogenase kinase 4; LXR, liver X receptor; ABCA1, ATP-binding cassette A1; SREBF1, sterol regulatory element binding protein-1; GSTP1, glutathione S transferase P1; JUN, jun oncogen. Values are means ± SEM, n = 21. The incremental areas under the curve (AUC) are shown in inserts. Different letters indicate a difference (p <0.05) between shakes at a given time. For a given test shake, a filled symbol indicates that the corresponding value differs (p <0.05) from baseline (0 h) value.

Ex vivo incubation experiments

To explore whether the observed *in vivo* effects on gene expression changes were direct effects of the increased different FFA concentrations in plasma, we performed *ex vivo* incubation of PBMCs with different fatty acids representing the main fatty acid types in the three shakes, i.e. palmitic acid as SFA, oleic acid as MUFA and docosahexaenoic acid as PUFA. Q-PCR of *ABCA1* in all four subjects revealed increases after SFA, and decreases after PUFA incubation. In 3 out of 4 subjects ABCA1 expression was increased after MUFA incubations. *SREBF1* showed a similar decrease after PUFA incubation, and increases in 3 out of 4 subject after SFA and MUFA incubations. These changes were comparable to changes in gene expression observed after consumption of the different shakes. (**Figure 6**).

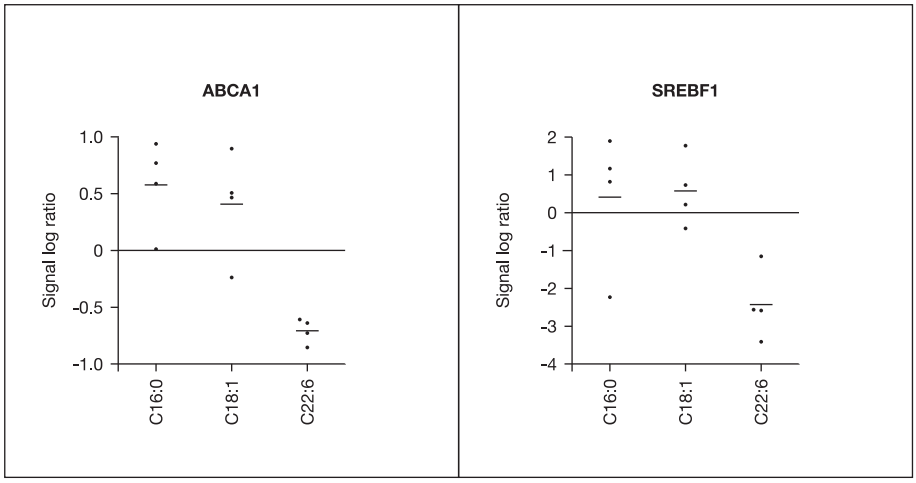

Figure 6 Effects of incubation of PBMCs from donors with three different fatty acids, which represent the main fatty acid types in the experimental shakes, on expression of several genes changed after shake intake. Values are means ± SEM, n=4. C16:0, palmitic acid; C18:1, oleic acid; C22:6, docosahexaenoic acid. ABCA1, ATP-binding cassette A1; SREBF1, sterol regulatory element binding protein- 1;

Discussion

This study describes the results of a single blind, cross-over trial, and demonstrates that consumption of high fat shakes varying in their fatty acid composition, results in postprandial fatty acid-specific gene expression profile changes in PBMCs. The difference in the gene expression profiles were characterized by differential effects on LXR signaling, glutathione metabolism, oxidative stress and inflammation.

For genes involved in LXR-signaling opposite effects of both shakes were found. The PUFA enriched shake decreased expression of genes involved in LXR signaling, whereas consumption of the SFA enriched shake resulted in an increased expression of the same genes. Most of these genes are target genes of LXR, and include *ABCG1*, *ABCA1* and *SREBF1*. Kinetics of the changes in expression of *SREBF1* and *ABCA1* in the hours following shake consumption revealed that differences between the two shakes were already present respectively 2 and 4 hours after consumption of the shakes and lasted up to 8 hours. This indicates a rapid response upon intake of fatty acids that disappears within several hours. The LXR target genes *ABCA1* and *ABCG1* play a major role in reverse cholesterol

transport in macrophages[137] and several *in vitro* studies have shown that PUFAs can decrease expression of these genes in macrophages[138, 139]. Our data is in line with these findings, because PUFA shake intake showed that expression of *ABCA1* and *ABCG1* was down-regulated and our *ex vivo* experiments showed that PBMC incubation with human DHA decreased *ABCA1* expression. However, the effect of dietary PUFA or SFA consumption on PBMC gene expression in humans has never been studied before and therefore the observed fatty acid-dependent response of *ABCA1* and *ABCG1* expression are completely new in that respect. Another target gene of LXR, *SREBF1* was also down-regulated upon PUFA intake and encodes a transcription factor involved in fatty acid synthesis and cholesterol metabolism[140]. Also for this transcription factor, no human *in vivo* experiments have been performed before, but *in vitro* studies have shown that PUFAs inhibit expression of *SREBF1* in hepatocytes[141] and embryonic kidney cells[142], which we also demonstrated in our *ex vivo* PBMC experiments after DHA incubation. Besides down-regulation of *SREBF1* upon PUFA shake intake, its target gene, *SCD,* was also down-regulated after PUFA shake consumption, whereas no change in regulation was found after SFA shake consumption. SCD is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids and expression of *SCD* was shown to be decreased by PUFAs through regulation of SREBF1 in animal and *in vitro* studies[143], which is clearly in accordance with our *in vivo* study findings. Yoshikawa *et al.* showed that PUFAs inhibit binding of LXR to LXR response elements in the promoter of LXR target genes such as *SREBF1*[142]. This repression of LXR target genes might also have happened in PBMCs of our *in vivo* study after consumption of the PUFA enriched shakes with a possible inhibition of cholesterol efflux and subsequent reverse cholesterol transport. Conversely, the increase in LXR target gene expression after SFA shake consumption might result in an increased cholesterol efflux out of the cells. However, it is hard to conclude whether SFA directly induce LXR activation, or whether cholesterol or its metabolites serve as ligands for LXR in these cells[144]. Since the SFA shake was made with butter fat, it contained more cholesterol than the PUFA shake. Moreover, we did not observe a clear increase in LXR target gene expression after *ex vivo* PBMC incubation with SFA. Also interesting in this respect is the observed decrease in expression of the low-density lipoprotein receptor (*LDLR*), which mediates uptake of LDL cholesterol in cells, after SFA shake consumption. *LDLR* is known to be an LXR target gene that is up-regulated after LXR activation[145], but

is down-regulated in our study, while other LXR target genes are up-regulated. On the other hand, rises in cellular cholesterol levels have shown to reduce transcription of *LDLR*[146], which might be the cause of our observed down-regulation in *LDLR* in human PBMCs after consumption of the SFA shake.

Another difference observed between consumption of both fatty acids was the response in cellular stress. PUFA consumption induced both an increased and decreased expression of genes involved in cellular stress response as illustrated by the up- and down-regulation of genes involved in NRF-2 mediated metabolism, whereas no effects were observed after SFA consumption. The down-regulated genes were involved in glutathione coupled metabolism, and mainly encoded glutathione S-transferases (GSTs), whereas the up-regulated genes were related to inflammation and cellular stress, such as *cJUN,* a component of activator protein 1, a transcription factor that regulates gene expression in response to a broad variety of stress stimuli. This bidirectional regulation of cellular stress points toward an initial triggering of the cells, and may work as a protective response mechanism of the cell.

The changed cellular stress response upon PUFA consumption shows that the use of a relatively high dose of DHA in the shakes is a suitable challenge strategy to induce cellular system perturbation, with the ultimate goal to reveal the resilience of the cells. Contrary, the PBMCs seemed to be capable of dealing with the high dose of the more common consumed saturated fatty acids with less difficulty, resulting in lower cellular stress responses. In addition, unlike the results of our postprandial study, long term high PUFA intake is capable of reducing stress in several studies[147, 148]. However, where long term interventions will provide information about the nutritional effects on whole body homeostasis, postprandial fat intake will provide information about the actual metabolic capacity of cells to deal with this increased lipid flow. For instance, prolonged intake of lower doses of DHA will probably increase the capacity of metabolic organs, such as intestine and liver, to more efficiently handle these potential stressors. This may result in an overall increased capability of the body to handle these lipids, and consequently lower stress response.

Within this study only one shake was consumed, but as we are in the postprandial state the largest part of the day, this raises the question what would happen with gene expression profiles, if several high fat challenges are given during the course of the day.

A interesting point to address is the low number of PPARα target genes which expression was changed after intake of the various shakes. Only a few PPARα target genes, including *PDK4, ADFP and SLC25A20* were found to be increased, and no differences were observed between the different shakes. PUFAs are known to be better ligands for PPARs then SFA and we therefore expected that PUFA shake intake would result in higher PPAR target gene expression then consumption of the SFA shake. In a previous study we observed that many more PPARα target genes were increased after 24 hour fasting than after shake intake in the current study, but we also found that endogenous derived plasma total FFA were much higher increased during fasting than after shake consumption. It seems that postprandial gene expression changes in human PBMCs are not regulated through PPARα or that other regulatory processes are more important in these cells.

This study is the first study that demonstrated that PBMC gene expression profiles can not only be used to differentiate between health and disease, as has been elucidated by previous whole genome gene expression studies[8, 10], but are also able to reflect postprandial changes that differ in type of fatty acid, only. It is known that the transcription of the cell will not change if the cell can handle the alteration in the environment. Therefore, challenge strategies combined with gene expression profiling of PBMC, as utilized within this study, can elucidated the capacity of cell systems to deal with stressors, such as the studied PUFAs, in a comprehensive manner. For future studies, it would be of interest to study if PBMC gene expression profiles reflect metabolic capacity upon fatty acid intake in subjects with diet-related disorders such as diabetes or metabolic syndrome, of whom the metabolic capacity is already retarded, and whether the responses would differ from healthy individuals.

In summary, our study shows that PBMCs are sensitive enough to show fatty acid-specific gene expression profiles after consumption of different fatty acids. Moreover, gene expression profiles of these cells can provide detailed information about cellular stressor handling capacity upon induction of a challenge. This makes these cells highly interesting for future utilization in the elucidation of the influences of nutrition on human homeostasis.

Chapter 6

General discussion

General discussion

The objective of this thesis was to elucidate the influence of nutrients such as fatty acids on whole genome gene expression profiles of peripheral blood mononuclear cells, which are circulating immune cells in the body. Our initial study was aimed as proof of principle in which we showed a marked change in PBMC gene expression profiles upon fasting, which elevates free fatty acid (FFA) concentrations in the blood, and demonstrated that the gene expression profiles of these cell can reflect this physiological condition in human individuals (**chapter 2**). In subsequent *ex vivo* PBMC studies we investigated the role of PPARα activation, of which several target genes were increased during fasting. We observed that gene expression changes after PPARα activation were regulated in a person specific manner (**Chapter 3**). This study indicated that it is possible to study PPARα in a comprehensive way in human PBMCs. After establishment of the role of PPARα in PBMCs, two nutritional intervention studies were performed which revealed that long term supplementation with polyunsaturated fatty acids (PUFAs) leads to changes in PBMCs that are characterized by an anti-inflammatory and anti-atherogenic gene expression profile (C**hapter 4**). The postprandial challenge study resulted in an change in cellular stress-related gene expression markers after consumption of PUFAs (**chapter 5**). Additionally, exact opposite postprandial effects of PUFA and SFA consumption on expression of LXR target genes were observed, demonstrating a reduced expression of these genes after PUFA intake and an increased expression after SFA intake.

Altogether these studies demonstrate that PBMC gene expression profiling is applicable in human dietary intervention studies. In addition, potential mechanisms underlying changes in gene expression after consumption of different fatty acids were revealed.

There are a few points to address when comparing data of all studies described in this thesis. The fact that the fatty acid-sensing nuclear receptor PPARα is expressed in human PBMCs was one of the arguments why PBMCs would be interesting cells to study fatty acid-dependent gene expression. Upon fasting, when FFA rise, several target genes of PPARα were increased. However, when we examined data of the nutritional intervention studies, expression of only a few PPARα target genes was changed. Since PUFAs are known to be better ligands for PPARs, we also expected that PUFA intake would result in increased PPARα

target gene expression compared with other fatty acids. Interestingly, for the long term PUFA supplementation study we did observe differences between the two fatty acid groups. However, instead of the expected increase, we observed that long term PUFA supplementation resulted in a down-regulation of PPARα target genes, while no such effect was observed for the MUFA group. In the postprandial study, PPARα target gene expression was increased, but no differences between the different fatty acid groups was observed. One of the likely reasons for the low number and direction of PPARα target gene changes in the nutritional intervention studies when compared with the fasting study, may be the difference in total plasma FFAs. After fasting, total FFA are extremely increased, with a consequent increased expression of a high number of PPARα target genes, whereas plasma total FFA are far less increased postprandially, with a consequent much lower number of PPARα target genes with an increased expression. Moreover, after long term PUFA intake, plasma total FFA are even decreased with a consequent decreased expression in PPARα target genes.
Besides the difference in PPARα target gene expression between the long and postprandial intervention, opposite effects were also observed on inflammatory gene expression profiles, which showed anti-inflammatory characteristics after long term PUFA intake, and more stress related responses, postprandially.

We hypothesize that during long term PUFA supplementation, when PUFAs are consumed regularly and in a low dose, the body could gradually adapt to the constant challenge of PUFA molecules that serve as excellent transcriptional activators through nuclear receptors such as PPARs. Moreover, this will directly or indirectly have effects on other transcription factors that are involved in lipid handling and inflammation, such as SREBPs, LXRs, FXRs, HNF4 or even NF-κB[149], which will lead to significant enhancement of metabolic functions of target organs such as intestine, liver, adipose tissue, endothelial system or heart. In the long run, this might contribute to the apparent positive effects found in the long term study. In contrast, saturated fatty acids (in particular C 16:0 and C18:0) that are more abundantly present in the normal western diet are less potent activators of the above mentioned transcription factors, or have even opposite effects, that may contribute to lower metabolic capacity and slightly pro-inflammatory conditions[150, 151]. Therefore, long term exposure to chronically high levels of saturated fat, as in a western style diet, characterized by low variability of food

patterns, would not sufficiently trigger the adaptability of organs, mediated in part by members of the nutrient sensing-nuclear receptor super-family, such as PPARs. This may in the end result in a less healthy phenotype of the individual. Hence, where long term fatty acid intake results in PBMC gene expression profiles reflecting more systemic adaptations of the body, postprandial profiles likely reflects more direct effects of the fatty acids on the cellular system. This acute effect may be explained by the fact that cellular gene expression will only change when the cellular system is not capable of handling the nutritional challenge. In this case, cellular regulators, such as transcription factors, will be activated and regulate gene expression to deal with the environmental changes. Conversely, when gene expression is not changed, apparently the cell is fully capable of handling the challenge. Hence, application of nutritional challenges such as described in chapter 5, will provide measures for cellular metabolic capacity.

Besides the studies described in these thesis, other studies have been performed that explored the feasibility of human nutrigenomics using circulating blood cells. Van Erk *et al*[152] examined the effects of a high-carbohydrate and a high-protein breakfast on gene expression of blood cells. However, paracetamol was added to the breakfasts to measure gastric emptying. Paracetamol is known to have profound effects on gene expression of peripheral blood cells[153] and might therefore have influenced the found results. In addition, in their study whole blood RNA isolation was used, which has been shown to increase noise and reduce responsiveness in gene expression profiles compared to the isolation of RNA from separated PBMCs[154]. Another study showed that a relatively strong intervention of 8-week low calorie diet decreased expression of oxidative stress and inflammation related genes in PBMCs[87]. To our knowledge, only one study examined the effect of fish oil supplementation on human leukocyte gene expression[95]. However, only 588 genes using a macroarray, instead of the more comprehensive microarrays used in our studies containing at least 2/3 of the human genome (17.699 genes), were examined in a pool of eight volunteers.

We are convinced that the studies that are described in this thesis contribute substantially to the field of human nutrigenomics and that the current knowledge on PBMC gene expression profiles is highly promising for future applications.

Gene expression profiles of these cells have clearly shown their potential and can bring a significant contribution to nutritional science in human subjects.

Future perspectives of human PBMC-based nutrigenomics applications

Within all studies described in this thesis, PBMCs were used as starting point for all analyzes. PBMCs, however, consist of different subpopulations and within the observed gene expression profiles characteristics were found that have been described for specific subpopulation. Each cell population within PBMCs has its own function and will therefore express cell type-specific genes[17, 155]. Isolation of the various subpopulations from blood is feasible, but still requires substantial amounts of blood and long isolation procedures, which introduce the risk of activating the cells, ensuing changes in transcription profile. Nevertheless, elucidation of fatty acid-specific effects on those subpopulation may facilitate the identification of molecular mechanisms of action of these fatty acids.

The research in this thesis focused on fatty acids or a synthetic counterpart, only. Future efforts must be taken to show whether interventions with other nutrients also results in gene expression profiles changes in PBMCs. However, it can be expected that other macronutrients, such as carbohydrates and proteins, or micronutrients, such as vitamins, which are also known to bind to and activate transcription factors, will alter gene expression profiles. In addition, effects of whole food based dietary intervention, in which different transcription activating nutrients are combined in food products, should be examined. Nevertheless, these studies are only the start in the elucidation of the effect on fatty acids on whole genome transcriptional profiling.

One of the interesting aspects described within this thesis is the individual response that was observed after an *ex vivo* challenge test (**Chapter 3**). These individual differences in PBMC gene expression profile response are often seen as a hindrance in the search for common responses or biomarkers. Conversely, these specific responses can be seen as a virtue, specifying each individual gene expression profile, enabling them to be used as personalized response fingerprints. Identification of the origin of these individual response profiles might bring us a step closer to realization of subject specific measurements with the ultimate goal to provide individuals with personalized dietary advise.

In addition, the differences in metabolic response upon the *in vivo* challenge test with different fatty acids might be useful to measure flexibility of the human system. Future research can further evaluate the power of this test for usages as a dietary consumption test or as a measure for health state. For instance, people with a western type diet, consuming hardly any fish, may respond different on a challenge of DHA than people consuming generally considered healthier diets that contain higher amounts of fish. In addition, people with diabetes may respond differently to a DHA challenge test than healthy subjects that should possess higher metabolic flexibility.

The ultimate goal is to identify individuals that are not sick yet, but are in the process of developing a diseases. This provides the ability to intervene with diet before the onset of the disease, in order to prevent instead of cure. Identification of such a "non-health" state requires also excellent phenotyping of the subject, that can be performed with advanced imaging techniques, such as magnetic resonance imaging (MRI), and functional tests, such as vascular function measures. In addition, in order to form a comprehensive set of information, besides transcriptomics, other genomic techniques, such as metabolomics and proteomics, should be utilized.

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Samenvatting

Achtergrond informatie

Nutrigenomics is de studie van de invloed van voeding op de expressie van genen. Het doel van nutrigenomics is om deze veranderde expressie van vele genen te vertalen naar veranderingen in fysiologische processen die de gezondheid beïnvloeden.

De mens heeft ruim 20.000 genen en de informatie van deze genen ligt opgeslagen op het DNA, dat in de kern van onze cellen te vinden is. Genen zijn de informatiedragers die ervoor zorgen dat er eiwitten worden geproduceerd in verschillende soorten cellen. Wanneer dat vereist is zal een gen afgelezen worden van het DNA en wordt er een kopie van dat gen gemaakt (transcriptie), in de vorm van messenger (boodschapper) RNA (mRNA). Deze kopieën worden vervolgens vertaald in eiwitten die een bepaalde functie in de cel of in het lichaam zullen gaan vervullen. Hoewel alle cellen in het menselijk lichaam hetzelfde DNA hebben, zullen niet alle genen in alle cellen altijd even actief zijn. Zo zullen er in cellen met verschillende functies ook verschillende genen afgeschreven worden. Bovendien kunnen er ook binnen dezelfde celsoort verschillende genen geactiveerd worden, afhankelijk van de omgevingssituatie en de processen in de cel. Genen kunnen dus "aan" of "uit" staan. Wanneer een gen "aan" staat en er dus mRNA wordt gemaakt, zeggen we dat een gen "tot expressie komt". De expressie van een gen kun je dus bepalen door de hoeveelheid van het bijbehorende mRNA vast te stellen.

Met behulp van hedendaagse technieken zijn we in staat om de expressie (activiteit) van bijna alle genen van de mens tegelijkertijd te bepalen. Dit doen we door gebruik te maken van zogenaamde microarrays. Deze microarrays zijn kleine glasplaatjes van 1 cm^2 waarop fragmenten van duizenden genen zijn gefixeerd. Wanneer we het mRNA uit cellen isoleren en deze op dit plaatje aanbrengen, zal dit mRNA vastplakken aan de bijbehorende fragmenten. Hierdoor weten we welk en hoeveel mRNA aanwezig is in onze cellen, en dus welke en in welke mate genen tot expressie komen in deze cellen. Op deze manier is het mogelijk om de genexpressie van cellen te vergelijken onder verschillende omstandigheden, bijvoorbeeld voor en na een maaltijd.

Van verschillende voedingsstoffen, waaronder vetzuren, is bekend dat ze de expressie van genen kunnen beïnvloeden. Vetzuren kunnen ingedeeld worden aan de hand van de mate van verzadiging en de lengte van de vetzuurketens. De meest voorkomende vetzuren in onze voeding zijn de enkelvoudig onverzadigde (in het Engels: monounsaturated fatty acids; MUFAs) en de verzadigde vetzuren (saturated fatty acids; SFAs). Meervoudig onverzadigde vetzuren (polyunsaturated fatty acids; PUFAs) komen in kleinere hoeveelheden voor, maar zijn relatief belangrijk, omdat het menselijk lichaam niet in staat is alle PUFAs uit andere vetzuren te maken. Omdat deze wel nodig zijn in het lichaam moeten we deze PUFAs binnenkrijgen via onze voeding.
Vetzuren beïnvloeden de expressie van genen door te binden aan bepaalde eiwitten in de cel. Deze eiwitten heten transcriptiefactoren, omdat ze de transcriptie (het maken van een kopie) van genen reguleren. Vetzuren binden aan de transcriptiefactor die PPAR wordt genoemd. Door deze binding wordt PPAR geactiveerd en worden genen afgeschreven die bijvoorbeeld betrokken zijn bij de regulatie van vetverbranding in cellen. Via de binding met PPAR reguleren vetzuren dus het proces van verwijdering van vetzuren in de cellen.

Omdat voor het bestuderen van de genexpressie cellen nodig zijn, is het moeilijk om dit soort onderzoek in gezonde mensen uit te voeren. Het is namelijk niet makkelijk om lever- of darmcellen te verkrijgen uit gezonde mensen. Bloedcellen zijn echter relatief gemakkelijk te verkrijgen via het afnemen van bloed. Bovendien is al gebleken dat witte bloedcellen, en dan met name de perifere bloedcellen lymfocyten en monocyten (zogenaamde PBMCs), gebruikt kunnen worden om verschillen in genexpressie aan te tonen tussen zieke mensen en gezonde mensen. Aangezien PBMCs een belangrijke rol spelen in het afweersysteem, heeft ziekte een vrij sterk effect op de genexpressie van deze cellen. Het was echter onduidelijk of de veel minder sterke effecten van voedingsstoffen op de genexpressie ook te detecteren zijn in PBMCs. De aanwezigheid van PPAR, de transcriptiefactor voor vetzuren, in PBMCs was een doorslaggevende reden om ons te richten op de effecten van vetzuren op de genexpressie van humane PBMCs.

Onderzoeksvraagstelling
Met het onderzoek dat beschreven staat in dit proefschrift wilden we bestuderen of veranderingen van type en hoeveelheid vetzuren in de voeding terug te zien

zijn in de genexpressie profielen van humane PBMCs. Vervolgens wilden we onderzoeken of we uit deze profielen konden afleiden welke invloed vetzuren hebben op de verschillende processen in PBMCs.

Resultaten

In het eerste onderzoek hebben we gekeken naar de effecten van twee dagen vasten op de genexpressie profielen in PBMCs van vier jonge mannen. Tijdens vasten nemen de vetzuurconcentraties in het bloed sterk toe. Daarom is deze situatie ideaal om te kijken of de genexpressie profielen van PBMCs ook veranderen. In dit onderzoek zagen we inderdaad veranderingen in genexpressie profielen van PBMCs na de vastenperiode. Veel genen waarvan de expressie veranderde bleken betrokken te zijn bij de vetverbranding. Daarnaast bleek dat veel genen waarvan de expressie veranderde, genen waren waarvan bekend was dat ze door PPARα, een subtype van PPAR, gereguleerd worden.

Daarom hebben we in een tweede onderzoek bestudeerd hoe groot de rol van deze transcriptiefactor PPARα was in PBMCs. Dit hebben we gedaan door PBMCs te isoleren uit het bloed van 6 bloeddonoren en ze samen met Wy14,643, een op vetzuren lijkend stofje, in een petrischaaltje te kweken en vervolgens de veranderingen in genexpressie te bepalen. Het voordeel van Wy14,643 ten opzichte van echte vetzuren is dat Wy14,642 alleen PPARα activeert, en geen andere PPAR subsoorten. Hierdoor weten we zeker dat de veranderingen die we vinden in de genexpressie van PBMCs na incubatie met Wy14,643, toegekend kunnen worden aan de activiteit van PPARα. In dit onderzoek vonden we dat PPARα de expressie van ongeveer 10% van de genen in PBMCs reguleert. Ook vonden we dat de 6 bloeddonoren na activatie van PPARα persoon-specifieke veranderingen in genexpressie profiel lieten zien. Na vergelijking van de resultaten van deze studie met de analyses van de eerste studie ontdekten we dat PPARα ook tijdens vasten een aanzienlijke rol speelt.

In de twee hierop volgende studies hebben we onderzocht of korte en lange termijn veranderingen in inname van verschillende vetzuren terug te zien zijn in PBMC genexpressie profielen. In de lange termijn studie hebben oudere mensen 6 maanden lang capsules geslikt met daarin ofwel visolie, dat meervoudig onverzadigde vetzuren (PUFAs) bevat, ofwel een controle olie met enkelvoudig onverzadigde vetzuren (MUFAs). We vergeleken de genexpressie profiel

veranderingen van PBMCs na 6 maanden visolie suppletie met de genexpressie profiel veranderingen na 6 maanden controle olie. Uit de studie bleek dat na het innemen van visolie met PUFAs de genexpressie een deels anti-stress profiel liet zien in PBMCs, terwijl je daar veel minder van zag na inname van de controle olie met MUFAs.

In de korte termijn studie hebben 21 jonge mannen drie verschillende milkshakes gedronken die alleen verschilden in het type vetzuur. Een milkshake bevatte PUFAs, MUFAs of verzadigde vetzuren (SFAs). We hebben genexpressie profiel veranderingen in de PMBCs van deze mannen, zes uur na inname van de milkshakes, vergeleken tussen de PUFA en de SFA milkshake. Verder hebben we voor enkele genen gekeken naar de genexpressie veranderingen in de tijd, tot 8 uur na de inname van de drie verschillende milkshakes. Uit de studie bleek dat inname van de PUFA-milkshake andere veranderingen in genexpressie profielen in PBMCs liet zien dan inname van de SFA-milkshake. Vooral cholesterol-regulerende genen kwamen meer tot expressie na de SFA-milkshake dan na de PUFA-milkshake. Metabole stress-gerelateerde genen kwamen juist meer tot expressie na de PUFA-milkshake, terwijl dat na de SFA-milkshake veel minder het geval was.

Wanneer we het lange termijn onderzoek vergeleken met het korte termijn onderzoek, dan valt op dat direct na inname van PUFAs meer stressgerelateerde genen tot expressie lijken te komen, maar dat na lange termijn inname van PUFAs juist het omgekeerd lijkt te gebeuren. Wij denken dat dit komt omdat PUFAs de cellen kunnen stimuleren, waardoor stressgerelateerde genen aangezet worden, maar dat dit er op de lange termijn voor zorgt dat de cellen beter met PUFAs om kunnen gaan. Met andere woorden, de capaciteit van de cellen om stress-inducerende factoren te verwerken neemt toe.

Conclusies

PBMC genexpressie profielen reflecteren veranderingen in vetzuurinname in de mens en zijn in staat om vetzuur-afhankelijke genexpressie patroon veranderingen te laten zien na inname van verschillende type vetzuren. Daarnaast kunnen we uit deze profielen processen afleiden die zouden kunnen veranderen in cellen naar aanleiding van de aanpassingen in de voeding.

Dankwoord

En dan nu, het belangrijkste onderdeel van dit boekje. Ik mag mijn naam dan voorop schrijven, maar het was zeker niet zover gekomen zonder een hele hoop anderen. In dit laatste stukje tekst wil ik dan ook graag mijn dank uitspreken voor al hetgeen zij voor mij hebben betekend...

Allereerst natuurlijk diegenen die dit alles mogelijk hebben gemaakt, en die mij in de laatste vier jaar vaak de weg hebben gewezen.

Michael, al begreep ik niet altijd direct welke kant je opging met je ideeën, door de vele discussies en de vrijheid die je me hebt gegeven, heb ik enorm veel geleerd de afgelopen vier jaar. Je werd gedurende die periode steeds enthousiaster over de resultaten en de mogelijkheden van het humane onderzoek en dat was een enorm goede motivatie voor mij. Hartelijk dank voor alles.

Lydia, je bent niet alleen een geweldig leuke 'baas', maar in de loop der jaren ook een voorbeeld als wetenschapper voor mij geworden. Jouw kritische, directe mening heeft mij meer dan eens wakker geschud, je uitgebreide kennis en inzichten hebben mij veel geleerd over de manier waarop wetenschap bedreven moet worden en ik heb ontzettend genoten van al onze uitgebreide discussies. Ik hoop dat we deze nog vaker zullen hebben, en aangezien ik nu slechts een steenworp verderop werk, zal dat vast en zeker het geval zijn!

Naast deze directe begeleiding waren er altijd anderen in de buurt waar ik terecht kon voor een vraag of met een probleem. **Guido**, bedankt voor je soms stevige commentaar, en al die momenten waarop je weer een array-analyse aan me uitlegde. Je was nooit te beroerd om me ergens mee te helpen, van het uitleggen van een computer programma, tot het meehelpen legen van mijn snoepla. **Sander**, je had vaak sterke kritische noten bij mijn werk, wat me erg motiveerde, en was altijd in voor wat ontspanning op de gang. Al moet je wel wat beter leren gooien…(ik kom niet terug om weer een bal van het dak te halen ☺). **Wilma**, je bent in veel dingen compleet het tegenovergestelde van mij (gestructureerd, georganiseerd, precies, duidelijk, etc), maar juist je kritische houding en opmerkingen hebben mij vaak enorm geholpen. Mijn dank daarvoor is groot!

Er zijn een hoop mensen die in hetzelfde (AIO-)schuitje zitten (of zaten) als ik zat, en ik wil hen allemaal even bedanken:

Rinke, als kamergenoot was het makkelijk om dingen snel aan je te vragen, en je had dan ook een grote hoeveelheid kennis waar ik vaak gebruik van heb gemaakt. Daarnaast hebben we ook aardig wat gelachen en ik zal nooit vergeten dat je zo vriendelijk was om de dief op onze kamer mijn portemonnee te wijzen en hoe ik bijna opgepakt ben omdat ik me uitgaf voor jou!! **Heleen**, ik kon altijd bij jou de kamer binnenlopen en een gezellig gesprek beginnen, even ontspannen en geintjes maken. We hebben veel lol gehad tijdens het klimmen, al kwam dat er de laatste tijd niet veel meer van. Bedankt voor het verduidelijken van vele zaken die een rol spelen in het AIO-leven, vooral tijdens mijn beginperiode. **Meike**, een goed gesprek en relativering kon ik altijd bij je vinden, hoe druk (en chaotisch) je ook bezig was. Jouw kennis was ook altijd zeer nuttig en gewoon even kletsen over iets anders was altijd fijn. **Jeroen**, we delen de liefde voor games en je tips en uitleen-games hebben me veel plezier en ontspanning gebracht. **Linda**, I always admired your calm and relaxed way of dealing with everything, and I really enjoyed your sense of humor on several occasions. **Anand**, thanks for teaching me about bayesian stuff and all those difficult computer things. You are by far the most friendly man I have ever met. Your great sense of humor and easy going mentality always make me feel good and I really enjoyed all the squash-games we played and probably will play in the future. **Fokko**, we deelden de gedrevenheid om het lekker laat te maken (al ben ik nooit door een raam naar buiten gegaan, ik had gewoon de code voor het alarm ☺). **David**, thanks for choosing me as your paranimph, and for the fun time we had organizing the PhD tour and actually being in the UK and Ireland. We had a great time, and I will never forget what we, together with Anand, found on top of the closet in that hostel! **Saskia**, je bent niet lang na mij begonnen en hoewel je wat tegenslagen te verwerken hebt gehad, geloof ik best dat het allemaal goed komt. Ik vond het altijd heel leuk om geintjes met (en over ☺) je te maken (het Limburgs leent zich er nu eenmaal uitstekend voor) en ik heb ook erg genoten van de squash-avonden. Laten we die voortzetten! **Laeticia**, besides the enormous amount of fun we had in our room, we also had some good discussions and you learned me a lot about this weird protein you love so much, FIAF (what did the letters stand for again?!). ...vous êtes com-

plètement taré! And off course, also thanks to your mum, who was so much fun during our trip to Marrakesh, even though we could not understand each other to well. (ce n'est pas jolie!). **David**, jouw rustige en goedlachse uitstraling waren een constante factor in de groep, en ook al hebben we weinig met elkaar te maken gehad, jouw vriendelijk lach was altijd welkom. **Natasha**, greek woman, you never stopped talking and was always hungry, two really funny qualities, which always made me laugh. You are a really positive person, and I am very happy to know you. **Susan** en **Diewertje**, jullie hebben altijd een lach op je gezicht en stralen van de positieve energie. Daarnaast doen jullie ontzettend leuk onderzoek, en is het nu aan jullie om Lydia scherp te houden! **Maryam**, you were very quite when you first joined as our roommate. But soon you understood our chaotic room-order, and it has been great fun to hear you behind me, trying to stay quiet, but not being able to stop yourself from laughing at one of the jokes being made. Not long after that you became one of the instigators of fun and I want to thank you for adding joy to the room. **Noortje**, bedankt voor je humor en gezelligheid in onze kamer. En natuurlijk voor de mogelijkheden die je me bood om je in te maken met squash (al lukt dat helaas niet altijd even goed...) Hopelijk zullen we dit nog lang voortzetten! **Zabi**, in de korte tijd dat je bij de groep hoort heb ik je nog niet goed leren kennen, maar je vriendelijke lach en gezellige groet doen me altijd goed. **Diederik**, je bent de nieuwste aanwinst in de groep en meteen iemand om rekening mee te houden. Momenteel bezig met de eerste grote proef, en ik hoop dat het allemaal voorspoedig zal verlopen. Bedankt dat jij tijdens de NWO dagen de plaats innam van diegene die volgens Sander bij mij op de kamer zou zitten... ☺!

Naast de gezellige samenwerking met de NMG-AIOs, heb ik natuurlijk veel meegemaakt met alle andere AIOs van de vakgroep Humane Voeding. Bij deze wil ik hen alle bedanken voor de leuke en gezellig tijd die ik heb gehad bij de vakgroep. Daarnaast wil ik met name de mensen bedanken die samen met mij de PhD-reis naar Engeland en Ierland hebben georganiseerd: **David**, **Renate**, **Pascalle**, **Marieke**, **Martinet** en **Gerda**. We hebben niet alleen veel geleerd van deze organisatie, maar ook enorm veel lol gehad. Ook alle mensen die mee waren op deze reis en de reis naar de Verenigde Staten, hartelijk dank voor alle lol en bus-quizen, en gezellige en leerzame discussies.

Natuurlijk zijn er nog vele anderen die mij op verschillende manieren geholpen hebben in de laatste vier jaar. **Shohreh**, you are the most helpful person I have ever known, and you were always there to lend a hand and help out when something had to be done, no matter how tired you were. Thanks a lot for everything! We will definitely eat some pizza again! **Mechteld**, allereerst is het natuurlijk helemaal geweldig dat je mijn paranimf wilde worden. Ik vind het echt fantastisch dat je samen met mijn zus naast me wilt staan op dat podium. Je stond ook altijd klaar de laatste jaren, en was altijd in voor een geintje. En natuurlijk heb je, samen met Jenny, al die microarrays voor me gedraaid. Helaas heb je me ook zover gekregen dat ik de Nijmeegse vierdaagse ben gaan lopen, maar, mede door jou, en natuurlijk ook **Menno**'s, instelling en gastvrijheid, ben ik erdoorheen gekomen. Bedankt voor al je hulp de laatste jaren, maar natuurlijk ook voor je gezelligheid, humor en gulle lach. **Jenny**, naast alle microarrays die je verwerkt hebt, nam je hulp met labwerk mij niet alleen enorm veel werk uit handen, vooral het laatste half jaar, maar wist ik ook zeker dat het in goede handen was. Je bent altijd enorm precies en kwam vaak met goede opmerkingen (waar ik dan natuurlijk weer helemaal niet aan gedacht had). Bedankt voor al je hulp en natuurlijk je gezelligheid. **Karin**, bedankt voor je herinneringen al de keren dat ik weer vergeten was dat het mijn beurt was om het lab schoon te houden (en dat kwam best vaak voor!) **Mieke**, altijd in voor een geintje, en natuurlijk voor een dansavond. Ik hoop je daar nog vaak aan te zullen treffen. **Carolien**, altijd een vriendelijke lach, zelfs als je 's avonds laat nog aan het werk was en ik verbaasd mijn hoofd om de deur stak omdat ik dacht alleen in het gebouw te zijn. **Els**, dank voor de ontzettend gezellige lunchgesprekken en natuurlijk ook bedankt voor het overhalen van Bas om mee te doen aan een van mijn sadistische voedingsproeven. Ik hoop dat hij me niet te onaardig vind ☺! **Jan**, altijd een gezellig praatje als ik je tegenkwam in de gang of op weg van of naar het Biotechnion, en natuurlijk bedankt voor al die mooie foto's die je door de jaren heen hebt gemaakt. **Nicole**, die goede oude Brabantse mentaliteit heb ik extra leren waarderen door jouw geweldige gezellige kijk op het leven. Vooral tijdens ons 'uitje' in Arizona hebben we veel gelachen en ik dank je dan ook voor je goedlachse humeur. **Jocelijn**, ik herinner me nog goed hoe we voor het filmpje voor Renger zo enorm gelachen hebben. Bedankt voor je oprechte interesse in mijn werk. **(Pappa) Bart**, dank voor je flauwe, maar vaak enorm grappige opmerkingen, en ik wens je veel succes toe met de gezinsuitbreiding. **Philip**, hartelijk dank voor de verwerking van de microarray data. **Anneke**,

maar weinig mensen beschikken over de vertelkunst die jij hebt laten horen en ik dank je dan ook hartelijk voor de vele lachbuien die je me bezorgd hebt. **Mark**, je was nooit te beroerd om even wat in Genomatix te gooien of ergens anders even snel naar te kijken. Hartelijk dank hiervoor, en ook voor het rechthangen van de schilderijen tijdens de NuGO week in Oslo ☺! **Roelof**, bedankt voor je kritische opmerkingen en je oprecht betrokken vragen bij een aantal van mijn projecten. **Renger**, bedankt voor je enorm positieve uitstraling, waardoor iedere vraag tijdens de donderdagochtend besprekingen zeer welkom was.

Hanneke, we hebben niet veel samen gewerkt, maar je was altijd degene die klaar stond als ik weer eens iets niet kon vinden op het lab. **Erik**, bedankt voor je gulle lach en humor tijdens de vierdaagse. Ik zal nooit vergeten dat ik rechts van je moet staan als ik een geintje ten koste van jou maak ☺! **Stephane**, thanks for your humor and for the help during my latest contacts with your lab. **Marleen**, je hebt een apart gevoel voor humor wat ik erg kan waarderen, en een geweldig relativerende kijk op het leven. Bedankt voor de leuke gesprekken die we op onze kamer gehad hebben. **Jolanda**, voor je op avontuur ging naar Spanje hebben we veel lol beleefd op onze kamer, waar ik toen "de nieuwe" was. Je hebt me meteen thuis laten voelen en me wegwijs gemaakt in de groep. Bedankt daarvoor en voor je gezellige gesprekken. **Betrand**, you were one of the people who was often present in the nights that I worked late, and you give one of the most cheerful greets I know. **Caroline**, I loved to make fun of your French accent, and you always cheered me up with another opportunity to do so. Thanks for returning the favor... **Pierluigi** and **Elvira**, thanks for your presence in the weekends in the last few weeks of all our contracts. It was nice to know I was not the only one working on impossible hours.

Ook enkele Master studenten hebben me geholpen tijdens het onderzoek en graag wil ik **Bianca**, **Ronak**, **Sonja**, **Antje** en **Neele** dan ook enorm bedanken voor hun inzet.

Naast alle genoemde mensen zijn er verschillende personen geweest die me bij mijn werk ondersteund hebben. Hiervan wil ik enkele met naam noemen, omdat ze een belangrijke bijdrage hebben geleverd tijdens mijn werk. Verschillende bloedpriksters hebben me geholpen tijdens de interventiestudies en met name

Lucy, **Jantien**, **Marga**, **Diana** en **Isabelle** wil ik bedanken voor hun inzet. Ook zonder diëtistes was ik niet ver gekomen in deze studies, en vooral **Els** wil ik bedanken voor haar inspanningen. Ook **Tineke**, **Betty** en **Truus** wil ik bedanken, voor het uitvoeren van enkele belangrijke labbepalingen. Daarnaast zijn **Dione**, **Lidwien**, **Marie** en **Gea** me meer dan eens te hulp geschoten. Dank voor jullie ondersteuning! Een beetje schone kamer draagt natuurlijk bij aan een mooi proefschrift en **Susan** deed jaren haar best om mijn altijd chaotisch bedekte bureau schoon te krijgen. Daarnaast dank voor het lenen van je scooter. Nogmaals excuses voor het te laat en niet geheel intact terugbrengen hiervan...

Graag wil ik **Baukje de Roos** bedanken voor het plaatsnemen in mijn promotiecommissie en het afreizen naar Wageningen, maar ook voor de bijzonder leuke tijd die we in Australië gehad hebben. I would like to show my gratitude to professor **Hannelore Daniel** and professor **Wim Saris** for their willingness to participate in my committee and to travel to Wageningen. Tevens wil ik hier professor **Huub Savelkoul** bedanken, voor het plaatsnemen in de promotiecommissie, maar ook voor het bieden van de mogelijkheid om verder te gaan in het onderzoek. Ik heb enorm veel zin om me de komende drie jaar in te zetten voor dit nieuwe project!

Zonder vrijwilligers kom je in het humane onderzoek niet ver, en ik wil dan ook mijn dank uitspreken aan alle **deelnemers** van de verschillende interventiestudies. Ik wil graag Professor **Gert Jan Hiddink** en de **Nederlandse Zuivel Organisatie** bedanken voor de financiering van dit project en de goede discussies die we gevoerd hebben tijdens de besprekingen.

I would like to thank all **NuGo members** for the lively discussions and the fun we had during the various meetings. Special thanks to professor **Sean Strain** for his nice words about me on various occasions, and to the **NuGies**, for all the fun we had during the last few years.

Johan, **Anne**, **Martijn**, die eigenlijk **Hendrik** heet (of was het nou andersom ☺) en **Martijn** (nu even wat jaren in de USA, maar we zien je vast wel weer eens terug), bedankt voor de heerlijk ontspannen etentjes en de grappige, telkens

weer nieuwe spelletjes. Ik beloof dat ik binnenkort meer tijd in mijn agenda heb om de gemiste afspraken in te halen!

Graag wil ik **Maarten** en zijn collega's van **Autobahn** bedanken voor het vele werk dat in het opmaken van dit boekje en het design van de kaft is gaan zitten!

Natuurlijk zou ik zonder de steun van mijn familie dit alles nooit bereikt hebben. **Ma**, welke keuze ik ook maakte in mijn leven, jij stond achter me. Je hebt altijd vertrouwen in mij gehad, en jij en **Dick** hebben me altijd gesteund. Dat heeft, geloof ik, goed uitgepakt ☺. **Pa**, al ben je niet goed in het uiten van je gevoelens, ik weet dat je enorm trots op me bent. Dit is altijd een sterke motivatie voor mij geweest en ik hoop je nog vaker trots te maken. **Inge**, ik vind het echt fantastisch dat je met mij op het podium wilt staan. Je bent altijd supertrots op mij geweest en **Jeroen** en jij, hebben altijd interesse getoond in mijn reilen en zeilen, en dat heeft veel voor mij betekend. **Oma**, helaas is **Opa** niet meer bij ons, maar ik wil jullie bedanken voor al de liefde en genegenheid die jullie mij in mijn leven gegeven hebben. Dit heeft er mede toe geleid dat ik hier nu ben aangeland.

Ook mijn schoonfamilie wil ik hartelijk bedanken. **Frans** en **Jifke**, **Maarten** en **Tanja**, **Ernst** en **Linda** en natuurlijk **Lars**, maar ook **Opa** en **Oma Oostveen**, vanaf het begin heb ik me thuis gevoeld bij jullie. Jullie zijn altijd enorm betrokken geweest bij alles wat ik deed, en dat geeft een zeer goed gevoel.

En dan mijn lieve meisie; **Carla**, zonder jou zou het er allemaal anders uitzien. Jij brengt een hele hoop liefde en geluk in mijn leven, en ik zou geen moment meer zonder je kunnen. Jij bent vaak mijn drijfveer, al ben je je daar zelden van bewust. Jij maakt mijn leven compleet en ik hoop dat ik je dat plezier terug geef.

Curriculum vitae

Mark Bouwens was born on the 30th of July, 1977, in Raamsdonk, the Netherlands. After completing secondary school in Raamsdonksveer at the "Dongemond college", he first intended to become a primary school teacher. However, after several years he decided to change course and focus more on his main interest in secondary school; biology. He graduated in 2001 from Fontys Hogescholen in Tilburg, the Netherlands, with a Bachelor's degree in Biology and qualified to teach at secondary school level. As a final project, he took on a job as a biology teacher at 'het Goese Lyceum' in Goes, the Netherlands, for 5 months as a maternity leave replacement. In that same year he started his university training in Biology at Wageningen University. Specializing in Molecular Biology, he performed study projects in the field of Virology and Immunology. He received his Master's degree in September 2004 and on the 4th of October that year he started his PhD project at the Nutrition, Metabolism and Genomics group of Wageningen University. This project's main goal was to elucidate the strength of peripheral blood mononuclear cells gene expression profiles to reflect nutritional effects in humans, and the project was funded by the Dutch Dairy Association (NZO). Results of this research are described in this thesis. During his PhD period he joined the educational program of the graduate school VLAG. Since January 2009 he has been working as a post-doctoral researcher at the Cell Biology and Immunology group of Wageningen University, led by Prof. Dr. Huub Savelkoul.

List of publications

Bouwens M, Afman LA, and Müller M. *Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid β-oxidation: functional role of peroxisome proliferator-activated receptor α in human peripheral blood mononuclear cells.* Am J Clin Nutr, 2007;86:1515–23

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Bouwens M, van de Rest O, Dellschaft N, Grootte Bromhaar M, de Groot CPGM, JM Geleijnse, Müller M and Afman LA. *Fish oil supplementation induces anti-inflammatory gene expression profiles in human blood mononuclear cells*

Bouwens M, Grootte Bromhaar M, Jansen J, Müller M and Afman LA. *Fish oil supplementation induces anti-inflammatory gene expression profiles in human blood mononuclear cells*

Afman LA, **Bouwens M**, Michalsen A and Müller M. *Changes in PBMC gene expression profiles of healthy subjects and subjects with the metabolic syndrome in response to a challenge of extreme caloric restriction*

Overview of educational activities

General courses

Career perspectives, 2008

Wageningen Graduate Schools, Wageningen, the Netherlands

PhD Competence assessment, 2007

Wageningen Graduate Schools, Wageningen, the Netherlands

Scientific writing, 2005

Centa language centre, Wageningen, the Netherlands

PhD Introduction week, 2004

VLAG graduate school, Bilthoven, the Netherlands

Specific courses

Molecular Mechanisms Involved in the Nutrient Control of Cellular Function, 2008

FASEB summer research conference, Arizona, USA

Minisymposium Nutrigenomics, 2006

Rank Price Funds, Lake district, UK

NuGO Advanced Microarray course, 2005

European Nutrigenomics Organisation, Maastricht, the Netherlands

NuGO Introduction week, 2005

European Nutrigenomics Organisation, Marseille, France

Scientific meetings

- PhD Study Tour to universities, research institutes and food companies in the USA (Boston, Ithaca, Pennsylvania, Philadelphia, Baltimore, Washington), 2007
- 4th Masterclass Nutrigenomics, 2005 (Wageningen, the Netherlands)
- PhD Study Tour to universities, research institutes and food companies in the UK and Ireland (London, Cambridge, Oxford, Reading, Dublin, Cork, Glasgow, Aberdeen, Dundee) (member of organizing committee), 2005
- Annual NuGO weeks, 2005 (Tuscany, Italy), 2006 (Oxford, UK) 2007 (Oslo, Norway)
- NWO Nutrition meetings, 2004, 2005, 2006 and 2008 (Arnhem and Deurne, the Netherlands)

- NZO research meetings, 2005, 2006 and 2007 (Wageningen, the Netherlands)
- Literature group "Journal Club", 2004-2008 (Organization: 2005-2007) (Wageningen, the Netherlands)
- Scientific discussion meetings NMG group, 2004-2008 (Wageningen, the Netherlands)

The research described in this thesis was financially supported by the Dutch Dairy Association (NZO).

Financial support from Wageningen University, the J.E. Jurriaanse Stichting, Promega Benelux and BD Biosciences for printing this thesis is gratefully acknowledged.

Cover design and lay-out
Autobahn, Utrecht, the Netherlands (www.autobahn.nl)

Printing
Grafisch bedrijf Ponsen en Looijen b.v., Wageningen, the Netherlands

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