Targeting PPARs in metabolic risk management: A pharmacological and nutritional approach

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**Targeting PPARs in metabolic
risk management:
a pharmacological and
nutritional approach**

Marjolijn C.E. Bragt-van Wijngaarden

The logo for NUTRIM, featuring the word "nutrim" in a lowercase, bold, sans-serif font. The letters are black, and the "i" has a dot.

The studies in this theses werd performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Foyal Netherlands Academy of Arts and Sciences.

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Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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*"Let als je naar een doel reist
goed op de weg.
Want de weg verrijkt ons
terwijl we hem bewandelen".*

*"When you travel toward an
objective, be sure to pay attention
to the road. It is the road that
enriches us as we walk its length."*

(Paulo Coelho)

Aan mijn ouders

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CHAPTER 1

General introduction

The global epidemic of obesity is clearly associated with the increasing incidence of diet-related diseases, such as cardiovascular diseases (CVD) and type II diabetes mellitus (T2D)¹. Obesity is often associated with various metabolic disturbances, such as abnormal lipid and carbohydrate metabolism and a pro-inflammatory state of the body. These lipid and non-lipid risk factors of metabolic origin predispose to the development of the metabolic syndrome (**figure 1**). The metabolic syndrome in clinical practice is most frequently diagnosed according to the guidelines of the National Cholesterol Education Program Third Adult Treatment Panel (NCEP ATPIII)². It defines the metabolic syndrome when 3 of the following criteria are present: waist circumference ≥ 102 cm in men and ≥ 88 cm in women, HDL cholesterol ≤ 1.03 mmol/L in men and ≤ 1.3 mmol/L in women, triglycerides ≥ 1.7 mmol/L and elevated blood pressure (systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg). These conditions increases the risk of developing CVD and T2D³.

The exact mechanisms underlying the metabolic syndrome are still unknown, but potential molecular targets to treat and prevent this metabolic disturbance are peroxisome proliferator-activated receptors (PPARs). PPARs are one of the central regulators of nutrient-gene interactions and involved in various metabolic processes. Understanding the physiological and molecular effects of PPARs in these processes will therefore provide insight in potential targets for the prevention of the metabolic syndrome

This chapter will give a general introduction to the metabolic processes involved in the metabolic syndrome and the role of PPARs herein and provides the outline of this thesis.

Figure 1. Metabolic disturbances and diseases associated with the metabolic syndrome.

1. Regulation and deregulation of lipid and lipoprotein metabolism

A large part of morbidity and mortality in the Western population is related to disturbances in lipid metabolism. Therefore, many attempts have been made in human studies and animal studies to modulate specific lipids and constituents of lipoprotein particles in order to understand and to prevent the consequences of these disturbances. This paragraph will briefly describe the regulation of lipid and lipoprotein metabolism.

1.1 Lipoproteins

Lipoproteins are particles found in plasma that transport the fat-soluble cholesterol and triglycerides. There are various lipoprotein classes: chylomicrons, very low density lipoprotein (VLDL), intermediate dense lipoproteins (IDL), low density lipoproteins and high density lipoproteins (HDL). Each class varies in size, weight, lipid composition, density and apolipoprotein content (**table 1**). Lipoproteins consist of a hydrophobic core containing esterified cholesterol and triglycerides, surrounded by a monolayer of phospholipids. The polar phospholipid surface allows the lipoproteins to travel in the aqueous blood plasma. The lipoprotein membrane also contains apolipoproteins, the major protein components of lipoproteins. Apolipoproteins are among others responsible for the recognition of the lipoprotein particle by a specific receptor. Importantly, within their class, lipoproteins are in a constant flux following a continuum of decreasing size and density. This means that within each class, lipoproteins can be further divided into subclasses⁴. For example, LDL can be divided in the subclasses light (LDL1 and 2), intermediate (LDL3), or dense (LDL4 and 5).

Table 1. Composition and properties of lipoprotein classes.

Lipoprotein class	Density (g/ mL)	Diameter (nm)	Protein (%)	Cholesterol (%)	Phospho-lipid(%)	Triglycerides (%)
Chylomicron	<0.95	100-500	1-2	8	7	84
VLDL	0.95-1.006	30-80	10	22	18	50
LDL	1.006-1.019	25-50	18	29	22	31
IDL	1.019-1.063	18-28	25	50	21	4
HDL	1.063-1.21	5-15	33	30	29	8

1.2 Lipid and lipoprotein metabolism

The digestion and absorption of dietary fat are part of the so-called exogenous pathway (**figure 2**). Dietary fat consists mainly of triglycerides (TG), which are insoluble in the aqueous environment of the gut. Due to the action of bile salts, fat is emulsified yielding mixed micelles to transport dietary lipids to the

intestinal cells and to make these lipids more vulnerable to be attacked by water soluble enzymes. By the action of pancreatic lipase (PL) in the intestine, TG are hydrolyzed to free fatty acids (FFA) and monoacylglycerol. Short chain fatty acids (≤ 12 carbons) will enter the circulation directly. Other fatty acids will be first esterified back to TG after absorption in the enterocyte. Then, TG will enter the circulation as chylomicrons containing apoB48, A-I and A-IV via the lymphatic system. In the circulation, apoCII becomes attached and serves as a cofactor for lipoprotein lipase (LPL) to hydrolyze TG into FFA in the capillary endothelium. The resulting FFA will be taken up by peripheral tissues to be stored as fat, oxidized to provide energy, or re-esterified to TG in the liver and transported back into the circulation as very low density lipoproteins (VLDL). After depletion of TG, chylomicron remnant particles will be taken up by the apoE receptor in the liver and removed from the circulation.

The synthesis and secretion of TG and cholesterol by the liver is defined as the endogenous pathway (**figure 2**). Free fatty acids bound to albumin in blood plasma are taken up by the liver and packaged as TG into VLDL particles together with apoB100, CII and E. TG and VLDL can also be synthesized from a surplus of glucose and amino acids in the fed state. VLDL undergoes hydrolysis by the action of LPL together with cofactor apoCII, resulting in a smaller IDL particle. Part of the IDL particles is converted to LDL by the action of hepatic lipase (HL) and another part will be taken up again by the liver via the apoE or LDL receptor (LDLR). LDL particles containing apoB account for the majority of serum cholesterol and can be taken up via LDL receptors in the liver or in other peripheral tissues. There is a large heterogeneity in LDL particles, due to differences in the amount of cholesterol per particle. In this respect, a differentiation can be made between the highly atherogenic small dense particles and the less atherogenic buoyant LDL particles. Especially small dense LDL particles are considered to be atherogenic, since they can more easily cross the capillary endothelium⁵ and are more susceptible to oxidation⁶. In the endothelium, cholesteryl esters from these particles can be taken up and accumulate in macrophages, resulting in the formation of foam cells. Together with an increase in inflammation, this contributes to an increased risk to develop atherosclerosis.

HDL is involved in reverse cholesterol transport (**figure 2**). It receives excess cholesterol from peripheral tissues to transport it back to the liver. Cholesterol and phospholipids are transferred to apolipoprotein A1 (apoA1), a process catalyzed by ATP binding cassette transporter A1 (ABCA1). This results in the formation of a nascent HDL particle, also called pre- β -HDL. Then, the transporter ABCG1 and the enzyme lysolecithin cholesterol acyltransferase (LCAT) increase the capacity of HDL to receive and esterify cholesterol, leading to a mature and more dense HDL particle. HDL cholesteryl esters will be taken up by scavenger receptor B1 (SR-B1) in the liver and used for the synthesis of bile acids. HDL cholesteryl esters can also be shuttled to other lipoproteins by the action of cholesteryl ester transfer protein (CETP).

Figure 2. Schematic overview of lipoprotein metabolism.

ABC: ATP binding cassette transporter, Apo: apolipoprotein, CETP: cholesteryl ester transfer protein, FFA: free fatty acids, HDL: high density lipoprotein, HDLR: HDL receptor, HL: hepatic lipase, HSL: hormone sensitive lipase, IDL: intermediate density lipoprotein, LCAT: lysocleithin cholesterol acyltransferase, LDL: low density lipoprotein, LDLR: LDL receptor, LPL: lipoprotein lipase, PL: pancreatic lipase, SR-BI: scavenger receptor class B member 1, TG: triglycerides, VLDL: very low density lipoproteins.

1.3 Dyslipidemia in the metabolic syndrome

A feature of the metabolic syndrome is a deregulation of lipid and lipoprotein metabolism, resulting in an atherogenic lipoprotein profile characterized by small dense LDL, decreased high density HDL, and increased TG⁷. The lipid overflow in the metabolic syndrome can lead to the accumulation of fat in non-adipose tissues, such as muscle, heart and liver, which contributes to the development of insulin resistance⁸. Normally, in response to a meal, our body will produce insulin. Insulin stimulates LPL and inhibits hormone-sensitive lipase (HSL)⁹ (figure 3). This will result in an increased triglyceride and fatty acid synthesis in adipose tissue and liver. The metabolic syndrome, however, is often associated with an insulin-resistant state. Insulin is overproduced, but tissues do not adequately respond to insulin anymore. This leads to lipid metabolic disturbances (figure 3). In the insulin resistant state, the inhibitory effect of insulin on HSL is diminished or lost, leading to increased activity of HSL. This results in an increased release of FFA from the adipose tissue⁹. Also, LPL will be inhibited in the insulin resistant

state resulting in the accumulation of TG-rich VLDL particles. TG from these VLDL particles can be transported via CETP to HDL, resulting in smaller TG-rich HDL particles. Small HDL a better substrate for HSL and therefore quickly cleared from the circulation¹⁰. As a consequence, less HDL particles are available for the reverse cholesterol transport. All together, this will ultimately lead to dyslipidemia, an important characteristic of the metabolic syndrome.

Figure 3. Schematic overview of lipid metabolism and disturbances in the metabolic syndrome.

FFA: free fatty acids, HDL: high density lipoprotein, HL: hepatic lipase, HSL: hormone sensitive lipase, IDL: intermediate density lipoprotein, LDL: low density lipoprotein, LDLR: LDL receptor, LPL: lipoprotein lipase, PL: pancreatic lipase, SR-BI: scavenger receptor class member 1, TG: triglycerides, VLDL: very low density lipoproteins.

2. Regulation and deregulation of glucose metabolism

Disturbances in lipid and glucose metabolism in the metabolic syndrome are inter-related. Excess of adipose tissue is associated with an increased secretion of insulin, leading to hyperinsulinemia. Insulin plays an important role in lipid metabolism, but is also the predominant regulator of glucose metabolism. Insulin lowers blood glucose by suppression of gluconeogenesis in the liver and stimulation of glucose uptake by peripheral tissues, mainly muscle and adipose

tissue. The metabolic syndrome is associated with resistance to insulin-mediated glucose uptake, and one of the diagnostic criteria of the metabolic syndrome is an elevated fasting plasma glucose level. As tissues become less sensitive to insulin, glucose and FFA levels will strongly increase after a meal, but the extra amount of insulin produced by the pancreatic β cells is still sufficient to maintain normoglycemia. However, at a certain moment, the pancreas will not be able to produce enough insulin to overcome the insulin resistance. In this case, gluconeogenesis in the liver will be strongly increased. Also, the inhibitory effect of insulin on adipose tissue HSL will disappear and causes FFA levels to further increase. In this way, hyperglycemia can develop, leading to the progression of metabolic syndrome into T2D or CVD.

3. Low-grade systemic inflammation

Metabolism and immunity are tightly linked, as many hormones, cytokines, signaling proteins, transcription factors, and bioactive lipids play a role in both metabolism and immunity¹¹. The metabolic syndrome is associated with a low-grade inflammation state¹², which plays a critical role in the development of insulin resistance, T2D¹¹ and CVD¹³. Body fat, especially visceral fat, is seen as a key regulator of inflammation. Adipose tissue is not merely a passive fat depot, but acts more as an active endocrine organ capable of secreting numerous cytokines. Adipocytes secrete a variety of pro-inflammatory proteins, such as MMP9, TNF α and IL6¹¹. It is also possible that these inflammatory proteins are secreted by macrophages, since these cells co-localize with adipocytes in adipose tissue and have overlapping metabolic and inflammatory signaling and sensing pathways¹¹. These secreted proteins contribute to a state of low-grade systemic inflammation. The increased level of pro-inflammatory cytokines together with elevated FFA levels in the plasma of obese subjects also stimulate the liver to secrete C-reactive protein (CRP)^{14, 15}. This even worsens the state of low-grade inflammation, because CRP is an acute phase protein involved in various inflammatory processes. As a result, the risk of developing T2D and CVD increases, as inflammation has been shown to be one of the underlying causes of atherosclerosis and insulin resistance^{16, 17}. However, it should be noted that body fat is not exclusively responsible for the state of low-grade inflammation. Other suggested contributors to the development of a low-grade inflammatory state are increases in nonesterified plasma fatty acids due to a decreased fat storage capacity in adipose tissue^{12, 18}, insensitivity to the anti-inflammatory effects of insulin^{12, 19}, or reduced anti-inflammatory molecules, such as adiponectin and IL10¹².

4. PPARs

A common denominator to link all metabolic and inflammation processes as described in the previous chapters is the involvement of peroxisome proliferator-

activated receptors (PPARs). PPARs are one of the central regulators of nutrient-gene interactions and are able to regulate lipid, carbohydrate, and inflammatory pathways, hereby maintaining homeostasis²⁰⁻²². So far, three PPAR isotypes have been identified in vertebrates: PPAR α , PPAR δ , and PPAR γ . PPAR α and PPAR γ are within the focus of this thesis and will be discussed in this paragraph. PPAR α is abundantly expressed in the liver and to a lesser extent in the kidney, testes, heart, small intestine, pancreas, smooth muscle and adipose tissue²³. PPAR γ mRNA is specifically expressed at high levels in adipose tissue, but is also detected in the intestine (colon), skeletal muscle, liver and bone marrow stromal cells²³. PPARs are ligand-activated transcription factors and activate transcription of their target genes as a heterodimer with their DNA binding partner retinoid X receptor (RXR)²⁴. The PPAR:RXR complex initiates gene transcription after binding to the PPAR response elements (PPRE) in enhancer sites of regulated genes (left part of figure 4).

Figure 4. PPAR signaling (left) and interference with NFκB signaling (right).

IκB: inhibitor κB, IKK: IκB kinase, NFκB: nuclear factor κB, PPAR: peroxisome proliferator-activated receptor, PPRE: PPAR response element, RXR: retinoid X receptor, ub: ubiquitination

4.1 PPAR ligands

The activity of PPARs can be modulated by synthetic ligands, such as the hypolipidemic drug fenofibrate (activates PPAR α) or the insulin-sensitizing drug rosiglitazone (activates PPAR γ). In addition, PPARs are able to sense the presence

of fatty acids²⁵. The types of fatty acids differ in their ability to activate PPARs, as has been demonstrated in studies using almost exclusively ligand-binding assays²⁴. In this respect, polyunsaturated fatty acids deserve attention as they have been shown to be very potent natural activators of PPARs^{26, 27}. There is a large overlap in ligand recognition between the PPAR isotypes²⁸, but regardless of this overlap, there will remain a functional difference between the isotypes due to differences in binding affinity of the ligands and the tissue distribution of PPARs²⁸.

4.2 Metabolic role of PPAR α

PPAR α is mainly involved in lipid metabolic processes to maintain lipid homeostasis. Increased plasma free fatty acid levels are thought to stimulate PPAR α target genes, resulting in an increased cellular uptake, intracellular esterification, and mitochondrial β -oxidation of fatty acids²⁹⁻³². PPAR agonists, like fibrates, improve the lipid profile by decreasing triglyceride levels and LDL cholesterol levels^{33, 34}. This hypotriglyceridemic effect is mediated by an increased lipoprotein lipase activity by PPAR α ³³. Additionally, a modification of plasma lipoproteins was observed after fibrate treatment, namely a reduction in the triglyceride content of VLDL particles and a reduction of small dense LDL particles³⁵. HDL cholesterol levels increase upon PPAR α activation, possibility via the induction of ATP binding cassette transporter A1 (ABCA1) or enhanced apolipoprotein A1 (ApoA1) mRNA expression^{36, 37}. In this way, PPAR α stimulates cholesterol efflux from peripheral tissues and subsequent uptake of cholesterol by the liver, as well as the release of new HDL particles into the circulation. All together, PPAR α activation is thought to contribute to a less atherogenic lipid profile and favors a healthier lipid homeostasis.

4.3 Metabolic role of PPAR γ

PPAR γ is mainly involved in glucose metabolism, but also exerts a potential beneficial effect on lipid metabolism³⁸. Studies with tissue-specific PPAR γ knockout mice, demonstrated that PPAR γ agonists exert their effects primarily by influencing skeletal muscle^{39, 40}. PPAR γ activation increases the expression of genes involved in glucose uptake and synthesis by skeletal muscle. Modulation of gene expression in liver will result in a reduced hepatic glucose production and subsequent release due to a decreased gluconeogenesis³⁹⁻⁴¹. PPAR γ also is a key regulator of adipocyte differentiation and adipogenesis. Stimulation of adipocyte differentiation via PPAR γ leads to the formation of an increased number of small adipocytes, which are able to take up free fatty acids more easily⁴². Consequently, the flux of free fatty acids to the liver and muscle will be decreased and reverses the insulin resistance of the muscle. This could ameliorate the effect of metabolic syndrome. In general, human intervention studies with PPAR γ agonists report reduced fasting insulin concentrations, glucose concentrations, and improved whole body insulin sensitivity³⁸. Like PPAR α agonists, PPAR γ agonists can reduce plasma free fatty acid concentration, mainly by increasing fatty acid

uptake and storage⁴³. Generally, HDL levels are increased upon treatment with a PPAR γ agonist⁴⁴. Differential effects have been observed for triglyceride and LDL cholesterol levels, depending on the type of PPAR γ agonist⁴⁵⁻⁴⁸. PPAR γ agonists are able to increase the size of LDL particles and decrease the number of atherogenic small dense LDL particles³⁸.

4.4 PPARs in inflammation

It is thought that PPAR α and PPAR γ represses inflammation by a physical interaction and forming inactive complexes with proinflammatory transcription factors, such as Nuclear Factor κ B (NF κ B) (**figure 4**)^{20, 49}. This transcription factor normally induces the transcription of pro-inflammatory genes, such as cytokines (IL6, TNF α), chemokines (IL8, MCP1), and cellular adhesion molecules (VCAM1, ICAM1)⁵⁰. Another way in which PPAR α and PPAR γ control inflammation is more indirectly by influencing transcription of genes that inhibit NF κ B signaling, such as Inhibitor κ B (I κ B) and I κ B kinase (IKK) (**figure 4**). For example, ligand-activated PPAR α is able to induce the expression of I κ B⁴⁹. This is a protein that binds to NF κ B and prevents the translocation of NF κ B to the cell nucleus and, subsequent, the transcription of pro-inflammatory genes. Therefore, induction of I κ B via PPAR α is correlated with a decrease in inflammation. Another example is the inhibition of IKK upon activation of PPAR γ ²⁰. Inhibition of IKK prevents the degradation of I κ B. In this way NF κ B stays bound to its inhibitor, retains in the cytoplasm, and is unable to activate gene transcription of pro-inflammatory genes. Due to this NF κ B inhibitory effect, targeting inflammation via PPARs may provide an interesting way to ameliorate the state of low-grade inflammation and reduce the risk of T2D and CVD.

5. Peripheral blood mononuclear cells as surrogate to study gene expression

Gene expression profiling is an interesting way to explore the effects of pharmacological or nutritional challenges on the metabolic state or condition of different target tissues. However, this is complicated in humans due to the relative inaccessibility of target tissues. Therefore, it would be advantageous to use blood cells, since blood is more readily accessible in contrast to muscle and adipose tissue for example. From a technical point of view, peripheral blood mononuclear cells (PBMCs) are very suitable to isolate from whole blood and for RNA extraction for the purpose of gene expression studies. PBMCs are cells with a round nucleus and consist of lymphocytes (T-cells, B-cells and NK-cells) and monocytes, which play an important role in immune responses.

Blood cells circulate through the body and may serve as sentinels, which pick up physiological and pathological changes and capture these changes by altering their transcriptome⁵¹. Gene expression studies have shown that PBMCs respond to nutritional⁻⁵², pharmacological⁻⁵³ and toxicological stimuli⁵⁴ and exercise⁵⁵. Furthermore, PBMCs have been used for diagnostic, mechanistic

and therapeutic assessments of different disease states⁵⁶. PBMCs also express PPARs, which makes PBMCs also interesting to study the molecular effects of these transcription factors. Altogether, studying gene expression in PBMCs can give more insight in the mechanisms behind the nutritional and pharmacological effects on several metabolic processes. This will contribute to the identification of early transcriptional biomarkers for the prevention of metabolic disturbances. However, more information is needed if PBMCs indeed give a good reflection of the metabolic condition of specific target tissues.

6. Outline of this thesis

This thesis provides insight in the physiological and molecular effects of PPARs in various metabolic processes and the role that nutrition can play in modulating these transcription factors. In this light, the suitability of PBMCs to study gene expression will be addressed. **Chapter 2** provides an extensive overview of the use of gene expression profiles in blood cells, with emphasis on the use of PBMCs, and discusses the applicability of these expression profiles to detect gene expression changes reflecting the condition of specific target tissues and metabolic changes. The role of PPAR γ in inflammation control is addressed in **chapter 3**. For this, we studied the effect of rosiglitazone on plasma inflammatory markers in T2D patients in the fasted and hyperinsulinemic state. In addition, we examined if changes in plasma inflammatory markers were also reflected by inflammation-related gene expression changes in PBMCs. In **chapters 4 and 5**, a comparison is made between a pharmacological (fenofibrate) and dietary (fish oil) approach to target PPAR α in a double-blind, randomized, placebo controlled, cross-over study in twenty overweight and obese subjects. First, the physiological effects of fenofibrate and fish oil on plasma and serum metabolic risk markers will be described (**chapter 4**). Secondly, the effects of fish oil and fenofibrate on gene expression profiles in PBMCs, muscle and adipose tissue are addressed (**chapter 5**). **Chapter 6** describes a mechanistic *in vitro* study to elucidate the role of PPAR α in mediating the effects of different fatty acids and fenofibrate on NF κ B activity in a human liver cell model. **Chapter 7** briefly summarizes the findings of the different studies that have been described. In this last chapter, the main results are discussed in depth and put into a broader perspective.

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CHAPTER 2

Blood-based gene expression analysis as a tool for biomarker discovery

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Abstract

Gene expression profiling can be used to understand the effects of external factors on the metabolic state or condition of specific target tissues. In humans, such studies are complicated by the inaccessibility of these tissues. Therefore, blood cells are used as a surrogate, because blood is more readily accessible. Blood cells circulate through the body and may serve as sentinels, which pick up physiological and pathological changes and capture these changes by altering their transcriptome. This characteristic of blood cells is interesting to understand underlying mechanisms and to identify transcriptional biomarkers of responses to external stimuli, such as nutrition, pharma, toxicological agents, and exercise. In this way, it may be possible to detect metabolic disturbances before a clinical event occurs. Furthermore, gene expression profiling in blood cells may also provide biomarkers for disease diagnosis, disease progression and survival, and the effectiveness of disease treatment. However, more information is needed before it will be possible to widely implement the use of gene expression profiling in blood cells into clinical practice. Little is known, for example, about inter- and intra-individual variation in blood cell gene expression. Furthermore, sample processing and the resulting complex mixture of cell types affect the gene expression profile, independent of the research question. The purpose of this review is to provide an overview of the use of gene expression profiles in blood cells, with emphasis on the use of Peripheral Blood Mononuclear Cells (PBMCs), and to discuss the applicability of these expression profiles to detect gene expression changes reflecting the condition of specific target tissues and metabolic changes.

1. Introduction

Gene expression profiling can be used to understand the effects of external factors, such as diet, on the metabolic state or condition of specific target tissues (**figure 1**). In humans, such studies are complicated by the inaccessibility of these tissues. Therefore, blood cells have been suggested as a surrogate, because blood is more readily accessible in humans. To be used as a surrogate, it is important that for the processes studied a relationship exists between the gene expression profiles in blood cells and those in the target tissues. More specifically, when studying changes in gene expression profiles as induced by an intervention, it is of importance that at the given dose and duration of exposure, the changes in gene expression profiles in blood cells correlate with those in the target tissue. Over 80% of the genes expressed in human blood cells are found in the brain, colon, heart, kidney, liver, lung, prostate, spleen or stomach. In addition, many of these genes are responsive to environmental changes¹. These findings contributed to the formulation of the so-called "Sentinel Principle", in which blood cells circulate as "sentinels" that respond to changes in the macro and micro environment of organs¹.

Gene expression profiling of surrogate cells or tissues can be very useful, not only to understand underlying molecular mechanisms, but also in the search for biomarkers that reflect early changes in gene expression caused by exposure to external stimuli. In this way, by using genetic biomarkers, it may be possible to already detect metabolic disturbances before a clinical event occurs. Surrogate tissue can also be useful for disease diagnosis, or for the prediction of disease progression and survival, or the effectiveness of disease treatment.

This review will give an overview of the use of gene expression profiles in blood cells in different settings, specifically focussing on Peripheral Blood Mononuclear Cells (PBMCs). In addition, the applicability of these blood cells as surrogate cells to detect gene expression changes reflecting the condition of specific target tissues will be discussed.

Figure 1. Gene expression profiling of PBMCs.

PBMCs circulate through the human body and are in close contact with human tissues, nutrients, toxic compounds and drugs. External and internal factors can thereby influence gene transcription in PBMCs. The use of RNA-based microarray experiments to obtain gene expression signatures can be a promising tool for disease diagnosis, predicting disease progression and response to an intervention.

2. Blood cells and gene expression profiling

2.1 Blood cell composition and blood processing

PBMCs are blood cells with a round nucleus and consist of lymphocytes (T-cells, B-cells and NK-cells) and monocytes. Other cells, present in whole blood, include red blood cells, platelets, reticulocytes, and granulocytes (neutrophils, eosinophils, basophils).

Blood cells for gene expression analysis can be isolated in different ways. RNA from whole blood can be isolated directly using the PAXgene™ (Becton Dickinson Company, Franklin lakes, NJ, USA) or QIAamp (Qiagen Benelux B.V., Venlo, the Netherlands) method. Ficoll-Hypaque density centrifugation and BD-CPT vacutainer tubes (Becton Dickinson Company, Franklin lakes, NJ, USA) can be used to isolate directly PBMCs from whole blood. It is important to realize that results can be affected by technical aspects, such as blood sampling, cell isolation procedures, time to analysis, and temperature during processing. Debey *et al.* investigated the influence of most of these technical aspects on gene expression profiles as determined with oligonucleotide microarrays in peripheral blood samples from 29 healthy blood donors². The choice of cell and RNA isolation technique (PAX, QIAamp, Ficoll-hypaque, BD-CPT) resulted in variable gene expression signatures. Significantly lower numbers of transcripts were found in PAX-isolated RNA samples from whole blood, which was likely due to the disturbing impact of reticulo-derived globin transcripts³. These transcripts are present in the red blood cells, which contribute to a large part to the amount of RNA isolated with this system. This suggests that the use of PBMCs is preferred

above the use of whole blood for gene expression profiling.

Temperature during processing had only a minor effect on the expression profile. The effect of temperature was reflected in the change in expression of mainly stress responsive genes, as confirmation with RT-PCR showed an increase in the expression of interleukin (IL) 8 and dual specificity phosphatase 1 (DUSP1) in Ficoll isolated samples at room temperature compared with samples processed at 8°C². Time delay (20-24h) to PBMC isolation and subsequent RNA extraction, however, had the largest impact on gene expression profiles, as evidenced by increased expression levels of early response and stress genes involved in the cell survival machinery². In addition, Molina *et al.* have demonstrated increased mRNA expression of cytokines in whole blood and PBMCs after delayed isolation of PBMCs from whole blood at room temperature⁴. Thus, these studies show that a rapid isolation of PBMCs is required to prevent changes in gene expression.

Not only isolation procedures, but also the relative proportions of cell types in blood determine gene expression profiles. The number of T cells, B-cells, NK cells, monocytes and granulocytes can vary greatly among individuals, depending on states of health and disease, and in response to stimuli⁵. In PBMCs, the proportion of monocytes can vary between 2-10%⁶, NK cells between 6-29%, T-lymphocytes from 61-85% and B-lymphocytes from 7-23%⁷. Palmer *et al.* investigated cell-type specific gene expression signatures for the major constituents of blood with DNA microarrays⁵. Clustering analysis could separate the samples in T-cells, B-cells, whole blood, and PBMCs, indicating cell-specific gene expression. Expression profiles of purified monocytes and NK-cells were not investigated. From the total T-cell population, profiles for CD4+ T cells and CD8+ T-cells could not be distinguished. In addition, genes characteristic for each cell sub-type were investigated. For the B-cell population, 427 genes were identified, that were mainly related to antigen binding, processing and presentation, and to MHC Class II receptor activity⁵. For T-cells, 222 specific genes were identified, which were related to their cell surface receptors and involved in T-cell receptor complex, signal transduction, and receptor activity⁵. Lymphocytes could be distinguished from granulocytes by the characteristic expression of 67 genes related to macromolecule biosynthesis and ribosomal proteins in lymphocytes, while cell surface and were mainly characteristic for granulocytes⁵.

To summarize, gene expression patterns are influenced by the choice of the cell isolation method and cell processing. It is crucial to rapidly process blood for RNA isolation, and to perform cell isolation preferentially at lower temperature to avoid effects on inflammatory and stress-related genes. Furthermore, one should also keep in mind that the proportion of subsets of cells can be influenced by the condition (health or disease) of a subject or by an intervention, which can influence the outcome of gene expression analysis.

2.2 Variation of gene expression in blood cells

To obtain insight into the normal variation between and within subjects, the variation in gene expression over time and variation associated with specific subject characteristics (such as age, BMI and gender), it is fundamental to understand the extent, nature and sources of variation in gene expression. To validate the use of blood cells as surrogate to detect potential changes in inaccessible tissues, information on the degree of variation in gene expression and responsiveness to environmental factors is essential. Independent of the research question, temporal variation, together with intra- and inter-individual variability, are important factors contributing to differences in gene expression patterns in blood cells. These sources of variation are especially important, when only small changes in gene expression are expected, such as in nutritional intervention studies in healthy subjects.

At least three studies have addressed inter- and/or intra-individual variation in expression profiles of blood cells from healthy volunteers⁸⁻¹⁰. Although these studies resulted in variable outcomes possibly due to differences in array platforms, genes represented on arrays, analytical procedures, and variation in leukocyte subsets, some important general conclusions can be drawn. Gene expression patterns in samples from the same individual were very consistent⁸⁻¹⁰. Eady *et al.* showed that intra-individual differences were relatively small, that is <20% for 80% of the 8489 genes examined⁸. This was confirmed by clustering analysis, in which samples from the same subject clustered together⁸. A cluster of 25 immunoglobulin genes showed the highest intra-individual variation. Differences in gene expression profiles between individuals were much larger compared to intra-individual differences⁸⁻¹⁰. 39% of the genes examined by Eady *et al.* differed significantly between sample sets from different donors and thus contributed to inter-individual differences in gene expression levels⁸. Individual specific gene sets, which overlapped between the studies of Eady *et al.*⁸, Radich *et al.*⁹, and Whitney *et al.*¹⁰, contained genes encoding for MHC class II molecules and interferon regulated genes. There were marked differences in gene expression between whole blood and PBMCs, at least partly due to differences in cellular composition of the samples¹⁰. Therefore, it is better to use PBMCs than whole blood to minimize variation between samples caused by differences in cellular composition. Other factors that can contribute to inter-individual variation are gender, age and BMI⁸⁻¹⁰.

Inter- and intra-individual variations are common in biological research and this information is, for example, needed for power calculations during the design phase of a study. The consistency of gene expression profiles in PBMCs over time within individuals is very promising for the application of microarray technology in intervention studies.

3 Blood cells as surrogate cells to monitor gene expression alteration in inaccessible target tissues

3.1 Blood cells and liver

In humans, the liver has an important role in coordinating and regulating lipid metabolism¹¹. There are several human and animal studies that have compared the expression of genes, involved in lipid metabolism, between PBMCs and the liver¹²⁻¹⁸.

As the LDL receptor and HMG CoA reductase play an important role in cholesterol metabolism, these two genes have been studied extensively. The LDL receptor is responsible for the uptake of LDL particles and HMG CoA reductase is the rate-limiting enzyme in endogenous cholesterol synthesis. Harwood *et al.* have suggested that in healthy men, the regulatory mechanisms in PBMCs controlling HMG CoA reductase activity are similar to those in the liver¹³. In that study, HMG CoA reductase activity in PBMCs was decreased during fasting or after a high-cholesterol meal and was increased by lowering circulating cholesterol levels. Similar changes in liver HMGCoA reductase activity have been demonstrated in animal studies^{12, 16, 18}. This was also confirmed at the transcriptome level by Powel and Kroon, who compared LDL receptor and HMG CoA reductase gene expression in livers and PBMCs of 13 patients who underwent cholecystectomy for symptomatic gallstones¹⁵. The number of LDL receptor mRNA copies per μg mRNA was equal between the liver and PBMCs, while in liver tissue the number of HMG CoA reductase molecules was less as compared to that in PBMCs. However, expression levels of both the LDL receptor and HMG CoA reductase in PBMCs closely paralleled expression in human liver, consistent with a coordinate regulation.

Other proteins involved in lipid metabolism are ATP binding cassette (ABC) proteins transmembrane transporter proteins, which facilitate the transport of lipids, sterols, drugs and large polypeptides. Expression levels of ABC transporter genes in PBMCs and liver were investigated by Moshen *et al.* in patients with hepatocellular carcinoma¹⁴. They found positive correlations for ABCA2, C1 and G1 between PBMCs and non-cancerous parts of the liver. ABCA2 and G1 are involved in cholesterol efflux, whereas ABCC1 plays a role in the development of multi-drug resistance by increased excretion of a drug out of the cell.

Vermunt *et al.* investigated the expression of various desaturases, which are important in the conversion of fatty acids, in the liver and in PBMCs of subjects undergoing a surgical operation¹⁷. The mRNA levels of $\Delta 6$ and $\Delta 9$ desaturase in PBMCs correlated negatively with those in the liver. However, no correlation was observed for $\Delta 5$ desaturase. These results indicate that $\Delta 6$ and $\Delta 9$ desaturase mRNA levels in PBMCs may predict those in the liver, although these genes may not be similarly regulated in both cell types.

Overall, it seems that for the limited number of genes studied, expression levels in PBMCs of genes involved in lipid homeostasis correlate with those in the liver.

3.2 *Blood cells and tissues relevant in toxicant exposure*

PBMCs could also play an important role as surrogate cells to measure toxicant exposure. The effect of toxicant exposure partly depends on the oxidative metabolism of toxic compounds, mediated by cytochromes P450 (CYPs) in the liver. These proteins are involved in detoxification, elimination, and activation of a wide variety of compounds. Furukawa *et al.* compared gene expression levels of CYPs in PBMCs and liver tissue from patients with hepatocellular carcinoma¹⁹. From the 19 CYPs measured, 6 genes could be detected in both PBMCs and non-tumour tissue from liver. From these genes, only the expression of CYP4B1 correlated between PBMCs and liver tissue, which indicate that PBMCs are not appropriate to study CYP expression in liver.

The relationship between the expression of early onco suppressor genes (*c-myc*, *Ha-ras* and *p53*) in rat PBMCs and potential target tissues (lung, liver, lymph nodes, kidneys and spleen) was investigated by Ember *et al.*, 24 and 48 hours after intraperitoneal exposure to the carcinogen 1-nitropene²⁰. The *c-myc* oncogene could not be used as biomarker, because of its low expression level in all tissues studied. Similar changes in gene expression patterns were only found between PBMCs and spleen, where early changes in *Ha-ras* and *p53* expression matched. No associations between gene expression changes with the other tissues were reported.

In view of the limited number of studies, in different species and with different research questions, it is not possible to conclude if genes in PBMCs could be useful as a molecular biomarker to study toxicological exposure in target tissues. More research in this area is certainly warranted.

4 Reflection of gene expression changes by blood cells

4.1 *Blood cell gene expression in nutrigenomics*

Gene expression profiling in blood cells has already been used as a tool to study the effects of dietary exposure in human intervention studies. Van Erck *et al.* evaluated the potential of this tool in a cross-over study investigating the effects of a high carbohydrate and a high protein breakfast in RNA from whole blood of 8 healthy men²¹. There were 176 differentially expressed genes in response to the high carbohydrate breakfast and 778 genes were changed after the high protein breakfast. The high carbohydrate intake resulted in lower expression of genes involved in glycogen metabolism, whereas the high protein intake resulted in lower expression of genes involved in protein biosynthesis. Furthermore, the high protein breakfast downregulated PGC1-related coactivator (PPRC1) and Lipin, genes involved in energy homeostasis and lipid metabolism. Fasting has also a pronounced influence on gene expression in blood cells. Bouwens *et al.* studied gene expression changes after 24 and 48h fasting in PBMCs of four healthy males²². A number of 1200 genes were changed after 24h fasting and 1386 genes after 48h fasting, with an overlap of >74% between both time-points. Pathway

analysis at these time-points revealed that fatty acid β -oxidation, pyruvate metabolism, and RNA-DNA metabolism pathways were upregulated, whereas the tricarboxylic acid cycle was downregulated. Fasting will increase plasma free fatty acid levels, because of the breakdown of triglycerides in the adipose tissue²³. These free fatty acids are able to activate nuclear receptor PPAR α , which can account for the observed effects, as expression of specific PPAR α target genes was strongly upregulated. The functionality of PPAR α in PBMCs was further demonstrated *in vitro*²⁴. From a physiological point of view, diet-induced changes in gene expression profiles can be expected to be much smaller compared to changes induced by drugs or disease. Though differences in gene expression profiles in PBMCs were not specifically related to those in other tissues, these studies clearly show the potential of using gene expression profiles in blood cells to understand diet-induced metabolic effects at the molecular level.

Most nutritional intervention studies have focussed on the transcriptional regulation of genes involved in cholesterol metabolism *in vivo*, in particular on LDL receptor²⁵⁻²⁸ and HMG CoA reductase^{13, 27, 28} mRNA expression in response to dietary changes. Boucher *et al.* showed that 7-day dietary cholesterol supplementation (791 ± 150 mg/d), after 4-day dietary cholesterol restriction (6 ± 4 mg/d), downregulated the expression of these genes in PBMCs of healthy human *in vivo*²⁷. They also measured LDL receptor-related protein (LRP), a receptor for the uptake of cholesterol-rich lipoprotein which plays a role in the regulation of serum lipoprotein levels²⁹. Upon cholesterol supplementation, the mRNA level of this receptor increased, which suggested a role of LRP for the protection against increased blood cholesterol levels. However, the exact function of LRP remains speculative. During cholesterol supplementation that followed the 4-day dietary fat restriction, there was a positive correlation between changes in LDL receptor and HMG CoA reductase expression. A study by Appelbaum-Bowden *et al.* also showed a downregulation of LDL receptor expression upon a high cholesterol diet (1034 ± 25 mg/d) versus a low cholesterol diet (137 ± 25 mg/d) in PBMCs of healthy individuals²⁵. Besides a response on gene expression, it has also been shown that PBMCs respond very quickly to changes in plasma and dietary cholesterol levels in term of enzyme activity. As shown by Harwood *et al.*, a single, high cholesterol meal decreased HMG CoA reductase activity within two hours, whereas cholesterol depletion by plasma pheresis led to a two-fold elevation in enzyme activity within 90 minutes of treatment¹³.

Functional foods, which influence cholesterol metabolism, have also been shown to reflect their effects on gene expression in PMBCs. Plant stanol ester consumption is known to inhibit intestinal cholesterol absorption and to lower plasma cholesterol in humans. Plat and Mensink showed that plant stanol ester consumption ($3.8 - 4$ g/d) increased LDL receptor mRNA levels in PBMCs from healthy men and women²⁸. HMGCoA reductase mRNA levels did not significantly change. However, mRNA levels of these two genes correlated positively in PBMCs, illustrating a coordinate expression of both genes. Another dietary component that may lower cholesterol levels is soy protein. The exact mechanism is unclear,

but effects may be due to the binding of isoflavones from soy protein to the oestrogen receptor. Baum *et al.* investigated the effects of soy consumption providing moderate (56 mg/d) and high (90 mg/d) amounts of isoflavones versus consumption of a control diet with casein and non-fat dry milk on lipoproteins and LDL receptor mRNA levels in hypercholesterolemic, postmenopausal women²⁶. Irrespective of the amount of isoflavones, serum total cholesterol levels did not change after soy consumption, but the LDL/HDL ratio decreased and LDL receptor mRNA increased in PBMCs.

To summarize, PBMCs respond to changes in plasma cholesterol by changing gene expression of LDL receptor and HMGCoA reductase. It appears that mechanisms underlying cellular cholesterol homeostasis overlap between the liver and PBMCs. This has frequently been demonstrated for the genes that regulate cholesterol uptake (LDL receptor) and cholesterol synthesis (HMG CoA reductase). In addition, these genes are regulated coordinately in PBMCs. Therefore, PBMCs are suitable biosensors to study diet-induced changes in LDL receptor and HMGCoA reductase expression in the liver.

4.2 *Blood cell gene expression in pharmacogenomics*

Gene expression profiles in blood cells can also be relevant to study the effectiveness of a specific treatment or to identify patients who respond most effectively to a treatment.

Atorvastatin, is a medicine used to treat dyslipidemia by lowering cholesterol synthesis via the inhibition of HMG CoA reductase activity in the liver. However, atorvastatin exerts also anti-inflammatory effects that may reduce the risk for cardiovascular, independent of its cholesterol-lowering effect. Wibaut-Berlaimont *et al.* investigated early and late effects of atorvastatin treatment on PBMC gene expression in dyslipidemic patients. For this, human microarrays containing 12,650 genes were used³⁰. The expression of 240 genes was changed upon atorvastatin treatment. Compared with baseline, 48 genes were differentially expressed after 12h, 75 genes after 36h, 58 genes after 1 week and 121 genes after 4 weeks. Many of these genes were involved in inflammation, hemostasis, or apoptosis, indicating the pleiotropic effects of this drug. Surprisingly, at the early time points (12h and/or 36h), expression of genes linked to the IFN α pathway, such as STAT1 (key signalling molecule downstream IFN α and IFN γ) and IL15 (monocyte cytokine) was increased. At these early time points, LDL receptor expression was also upregulated, although no significant effects on serum lipids levels were evident. There were also a number of downregulated pro-thrombotic genes, such as prostaglandin G/H synthase and the thromboxane A2 receptor. At late time-points (1 and/or 4 weeks), when serum total and LDL cholesterol levels were decreased, in particular expression of genes involved in apoptosis, such as the pro-apoptotic genes Bax and XIAP (apoptosis inhibitor) was downregulated. In conclusion, the difference in response on gene expression over time indicated both lipid-dependent and lipid-independent effects of atorvastatin treatment on gene expression. These results also indicate that PBMCs can be used as surrogate

to study drug response, which can lead to new insights into the mechanism of action of atorvastatin.

Transcriptional profiling of blood cells can also contribute to the process of drug development, for example to the development of Liver X Receptor (LXR) agonists. These agonists are of interest due to their anti-atherosclerotic effect, because they increase the expression of ABCA1 and ABCG1 resulting in an increased cholesterol transport. Peripheral blood cells express LXR α and LXR β and respond to LXR agonists by increasing the expression of these target genes in a time- and dose-dependent manner³¹. Therefore, these relevant target genes may be useful biological indicators for clinical development of LXR modulators³¹.

In cancer research it is important to find gene expression changes that can serve as biomarkers of drug activity. DePrimo *et al.* conducted a study with PBMC samples from 2 randomised, open-label, multi-centre phase III clinical trial to examine gene expression changes after treatment with standard chemotherapy or standard chemotherapy plus SU5416 (a vascular endothelial growth factor receptor kinase inhibitor) in patients with advanced colorectal cancer³². Expression profiling using Affimetrix microarrays revealed that 13 transcripts were specific to patients receiving SU5416 treatment. Of these transcripts, 6 were further tested in 62 additional samples. This confirmed that 4 of these genes, CD24, lactoferrin, lipocalin and MMP-9, consistently correlated with SU5416 treatment. Using the expression of these genes, it was possible to predict if the patient belonged to the SU5416 or control treatment arm. However, these four transcripts may merely represent biomarkers of SU5416 administration rather than a response to changes in disease status³².

A study by Hakonarson *et al.* is a good example of a pharmacological study that used gene expression profiles for identification of biomarkers of therapeutic benefit³³. Gene expression profiling in PBMCs of asthma patients was performed to identify genes that could predict responders and non-responders to inhaled glucocorticosteroids. Gene expression analyses revealed 15 genes that could predict response to glucocorticosteroids with 85% accuracy. Of these 15 genes, 11 genes could be confirmed and detected with Real Time PCR. Interestingly, the downregulation of the NF κ B gene predicted the response with 81% accuracy. Downregulation of NF κ B activation results in the reduced expression of inflammatory mediators, such as cytokines and chemokines, which play an important role in asthma. In addition to NF κ B, STAT4 and IL4R were also strong predictor genes. Whether this panel of genes are only reactive to the glucocorticoid treatment itself or also play a regulatory role in mediating the effects of glucocorticoid treatment in asthma remains to be established.

In conclusion, these encouraging results form a good basis to further explore the use of PBMCs as a surrogate system for biomarker analysis in pharmacology studies. However, future studies should not only focus on biomarkers of solely drug exposure, but more importantly, biomarkers should also reflect the mechanism of action of the drug and change in disease status upon drug treatment.

4.3 Blood cell gene expression in toxicogenomics

To study the early effects of exposure to a xenobiotic on gene expression, Van Leeuwen *et al.* exposed PBMCs from different donors *ex vivo* to cigarette smoke and its constituents for 18h³⁴. After exposure, gene expression was measured using a microarray containing 600 toxicologically relevant genes. Many genes involved in immune and stress responses were changed upon exposure. Other studies also showed gene expression changes in these processes after exposure to cigarette smoke, ionizing radiation, arsenic exposure and benzene exposure³⁵⁻³⁸. Surprisingly, Van Leeuwen *et al.* found no regulation of genes involved in DNA damage response and only a few involved in DNA repair³⁴. The gene that showed the largest effect was SERPINB2, a primary regulator of plasminogen activation, which inhibits apoptosis. In addition, *in vivo* research by Lampe *et al.* identified predictive signatures in PBMCs, which could specifically and sensitively distinguish smoking from non-smoking individuals³⁶. A subset of predictive genes (e.g. CYP1B1, IL1 β) also correlated with plasma levels of cotinine, the primary metabolite of nicotine. Thus, these results indicate that PBMCs are able to reflect toxicological relevant gene expression changes in response to cigarette smoke.

Cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1, respectively) are phase I enzymes, which are involved in the metabolic activation of dioxin-like compounds and the oxidative metabolism of estrogens. These enzymes can be found in many tissues, including PBMCs. Van Duursen *et al.* exposed PBMCs from 10 non-smoking healthy women *ex vivo* to dioxin-like compounds, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and the less potent dioxin-like polychlorinated biphenyl 126 (PCB126)³⁹. However, there was a large inter-individual variability in constitutive and induced CYP1A1 and CYP1B1 expression in lymphocytes³⁹. In addition, concentrations of dioxin(-like) compounds at which significant effects on gene expression were observed in PBMCs *in vitro*, were 10-fold higher as compared with levels found in human blood³⁹. Therefore, CYP1A1 and CYP1B1 might not be applicable as biomarkers of exposure to dioxin and dioxin-like compounds.

Rockett *et al.* compared gene expression changes in rat PBMCs and uteri following 17 β -estradiol exposure⁴⁰. They showed that of the 1185 genes measured, 193 genes were detectable in both tissues and 18 of these genes were significantly altered in both tissues. Of these 18 genes, 8 genes (e.g. jun-D, phospholipase A2, thymidine kinase) appeared to be treatment specific and not tissue specific, because gene expression changed in a similar degree and in a similar direction in both tissues⁴¹.

Studies describing the effects of different toxic compounds on gene expression changes in PBMCs did not suggest that a common set of genes can be used as a generic biomarker to measure exposure to toxic compounds. Furthermore, as also holds for pharmacogenomic research, it is important that future gene expression studies in PBMCs not solely searches for profiles and genes that reflect exposure, but also for profiles that reflect a biological response to the exposure.

4.4 Gene expression in blood cells in response to exercise

Exercise leads to an increase in circulating PBMCs and to an increase of both circulating pro-inflammatory and anti-inflammatory mediators⁴². Connolly *et al.* examined if these changes were paralleled by alterations in gene expression in PBMCs after an acute bout of exercise⁴³. For this, 15 healthy adult men (18-30y) performed 30 minutes of constant work rate cycle ergometry (~80% peak O₂ uptake). Gene expression was analysed pre-exercise, end-exercise and 60 minutes in recovery. Comparing the pre- and end-exercise gene expression profiles, revealed (mainly upregulated) changes in 311 genes, related to stress and inflammation. The expression of these genes returned to baseline after 60 minutes of recovery. Furthermore, IL1 receptor antagonist and IL6 inhibitor, both anti-inflammatory mediators, increased when comparing end-exercise and recovery profiles. Besides genes involved in inflammation, exercise also caused an upregulation of genes involved in growth and repair. However, it cannot be excluded that some of these gene expression changes in PBMC were due to alterations in the proportions of lymphocytes and monocytes in the circulation due to the exercise intervention⁴³. Comparable exercise-responsive genes were identified in a study conducted by Zieker *et al.* (CD14, chemokine ligand 4, CSF3R, HDPB1, IL1 receptor type 2, IL1 receptor antagonist, IL2 receptor beta)⁴⁴. In this study, gene expression was analysed in whole blood obtained from 8 half-marathon runners, before (t0), immediately after (t1) and 24 hours after exercise (t2). The genes represented on the custom-made microarray (384 cDNAs in triplicate) were selected based on their role in inflammation, apoptosis and stress responses. Cluster analysis revealed that 36 genes were changed comparing t1 with t0, and 21 genes were changed when t2 was compared with t0. All changes were in the same direction in all 8 half-marathon runners. This study showed that (exhaustive) physical activity is associated with a different pattern of gene expression at each time-point in response to a half-marathon.

Endurance training leads to adaptation in oxidative metabolism in skeletal muscle, as shown by a relative decrease in carbohydrate utilisation and increase in fatty acid oxidation⁴⁵. Zeibig *et al.* investigated whether these adaptive changes in skeletal muscle were also reflected in PBMCs⁴⁶. They studied the expression levels of carnitine palmitoyl transferases 1 and 2, which transport fatty acids into the mitochondria, and expression levels of carnitine acetyl transferase (CRAT), human specific microsomal carnitine acyltransferase (GRP58), and the organic cation transporter OCTN2, which are all involved in the uptake of L-carnitine across the plasma membrane in muscle and blood cells. Gene expression analysis was conducted in muscle and PBMCs of 6 endurance-trained subjects, before and after a 6-months training period with an average low-intensity training of 12h per week, consisting of running, cycling and skating. There was a concordant stimulation of CPT1, CPT2, CRAT, GRP58 and OCTN2 in PBMCs and muscle tissue. In addition, CPT1A, the liver isoform of CPT1, which is not expressed in adult skeletal muscle, was also increased to the same extent as the muscle isoform CPT1B in PBMCs. Thus, PBMCs can mimic gene expression changes that occur in muscle as a result

of endurance training.

Overall, examining the genomic response of blood cells to exercise could reveal mechanisms that can explain the metabolic effects of different forms of training. Furthermore, gene expression fingerprints might serve as an interesting research tool for monitoring exercise and training loads and for diagnosis and treatment of exercise related stress.

5. Gene expression profiles in blood cells for disease diagnose and prediction of disease progression

Variation of PBMC gene expression signatures observed in healthy subjects is markedly smaller than that observed among patients, and thus provides robust support for using gene expression patterns for characterisation and diagnosis of disease states⁴⁷. Research to identify specific genes in PBMCs, which reflect physiological and pathological state of different tissues of the body, will help the clinical practice in disease diagnosis and prediction of disease progression of patients. Tang *et al.* conducted one of the first studies in rats to demonstrate that gene expression profiles in blood cells could serve as fingerprint of different disease states, such as stroke, seizures, hypoglycaemia and hypoxia⁴⁸. Their results suggested that gene expression profiles in PBMCs had potential to be used for diagnostic, mechanistic and therapeutic assessment of different disease states. This approach has now also been implemented in human studies.

Blood cells can be representative surrogate cells to study multifactorial diseases, such as cardiovascular diseases (CVD) or type II diabetes. Gene expression analysis has been shown to discriminate patients with hypertension⁴⁹,⁵⁰, atherosclerosis⁵¹, and coronary artery disease patients⁵² from healthy controls. Another study with type II diabetic subjects showing the diagnostic potential of gene expression analysis in PBMCs, revealed that genes involved in the JNK pathway may be candidate markers for hyperglycemia-induced stress, whereas genes from the OXPHOS pathway may be markers of morbidity of type II diabetes⁵³. Gene expression analysis in PBMCs is not only useful for disease diagnosis, but may also help to unravel the aetiology of a disease. For example, PBMCs respond to changes in plasma lipid levels by regulating genes involved in lipid and fatty acid synthesis and inflammation, which can shed more light on the relation between plasma lipids and the development of diseases, such as atherosclerosis and CVD⁵⁴.

In the field of oncology, gene expression profiling in target tissues revealed differences between signatures from normal and malignant tissues^{55, 56}. In search for markers in surrogate tissue, Twine *et al.* identified a set of eight mainly inflammatory related genes, which distinguished PBMCs from advanced renal cell carcinoma patients and healthy volunteers⁵⁷. In addition, later research in this field even showed that gene expression patterns in PBMCs of patients with renal cell carcinoma correlated with disease prognosis⁵⁸. Not only gene expression target tissue, but also in blood cells, as surrogate cells, can be influenced by the early stages of breast cancer development. Using gene expression analysis of

1368 genes in women with breast cancer and women with no signs of the disease, Sharma *et al.* identified a gene set of 37 genes in RNA from whole blood, which predicted the existence of breast cancer in more than 80% of the cases⁵⁹. Distinct transcriptional profiles in blood cells were also identified for other types of cancer, such as bladder, colorectal and prostate cancer⁶⁰.

For many other diseases, the use of gene expression profiling may have potential for disease diagnosis, prognosis and progression, as has been suggested for haematological diseases, neurological disorders, psychiatric disorders and auto-immune diseases⁶⁰. The clinical applicability of gene expression profiles in blood cells as surrogate cells in predicting disease prognosis and progression was illustrated by Deng *et al.*⁶¹. They demonstrated the clinical potential of gene expression profiles in PBMCs by the use of a predictive gene panel to identify recipients with moderate or severe rejection of an allograft transplant from patients who tolerated the transplant^{61, 62}. Thus, the development of gene-expression based tests seem promising, although subsequent additional research is warranted for thorough validation and evaluation of cost-effectiveness of these assays in clinical practice.

6. Conclusion and future perspectives

At this moment, the application of blood transcriptomics is still in its infancy, although more and more studies are performed to identify biomarker genes related to responses to interventions, disease diagnosis, and prediction of disease progression. Overall, as sentinels that serve as an individual's health sensor, gene expression profiles in blood cells are able to reflect changes caused by diet, medicine intake, toxicant exposure and exercise. When studying gene expression in blood cells, standardisation of study procedures is important, including the choice of blood cells and RNA isolation technique and a strict control of blood handling time and temperature. Furthermore, the use of (specific) PBMC cells is preferred above the use of whole blood, to prevent the disturbing impact of reticulo-derived globin transcripts and granulocytes on gene expression. Challenges in future will be a) the selection of the most responsive cell population, as variation in amounts of specific cell types can influence gene expression profiles regardless of the question studied, b) well-controlled gene expression studies with well-characterised populations to decrease variation due to confounding variables (e.g. environmental factors) c) large trials specifically designed to validate the use of possible biomarkers, d) translation of gene expression profiles to the clinical practice, which include the study of associations between gene expression with relevant and objective clinical endpoints, the clinical applicability of expression assay, and the evaluation of cost-effectiveness. In future, analysis of candidate genes selected from microarray studies in PBMCs can potentially provide an efficient and sensitive tool to be implemented for routine use in the clinical practice and may provide additional information beyond already existing traditional biomarkers.

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CHAPTER 3

Anti-inflammatory effect of rosiglitazone is not reflected in expression of NFκB-related genes in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus

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Abstract

Rosiglitazone not only improves insulin-sensitivity, but also exerts anti-inflammatory effects. We have now examined in type 2 diabetic patients if these effects are reflected by changes in mRNA expression in peripheral blood mononuclear cells (PBMCs) to see if these cells can be used to study these anti-inflammatory effects at the molecular level *in vivo*. Eleven obese type 2 diabetic patients received rosiglitazone (2×4 mg/d) for 8 weeks. Fasting blood samples were obtained before and after treatment. Ten obese control subjects served as reference group. The expression of NFκB-related genes and PPARγ target genes in PBMCs, plasma TNFα, IL6, MCP1 and hsCRP concentrations were measured. In addition, blood samples were obtained after a hyperinsulinemic-euglycemic clamp. Rosiglitazone reduced plasma MCP1 and hsCRP concentrations in diabetic patients (-9.5 ± 5.3 pg/mL, $p=0.043$ and -1.1 ± 0.3 mg/L $p=0.003$), respectively). For hsCRP, the concentration became comparable with the non-diabetic reference group. However, of the 84 NFκB-related genes that were measured in PBMCs from type 2 diabetic subjects, only RELA, SLC20A1, INFγ and IL1R1 changed significantly ($p<0.05$). In addition, PPARγ and its target genes (CD36 and LPL) did not change. During the clamp, insulin reduced plasma MCP1 concentration in the diabetic and reference groups ($-9.1 \pm 1.8\%$, $p=0.001$ and $-11.1 \pm 4.1\%$, $p=0.023$, respectively) and increased IL6 concentration in the reference group only ($23.5 \pm 9.0\%$, $p=0.028$). In type 2 diabetic patients, the anti-inflammatory effect of rosiglitazone is not reflected by changes in NFκB and PPARγ target genes in PBMCs *in vivo*. Furthermore, our results do not support that high insulin concentrations contribute to the pro-inflammatory profile in type 2 diabetic patients.

1. Introduction

Rosiglitazone, an agonist for the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ), is a widely used drug for the treatment of type 2 diabetes mellitus. It belongs to the group of thiazolidinediones (TZD) and increases insulin sensitivity of peripheral tissues. In addition, there is evidence that rosiglitazone has anti-inflammatory effects^{1,3}. It has been postulated that rosiglitazone exerts its anti-inflammatory effect through inhibition of the transcription factor nuclear factor κ B (NF κ B) pathway¹. Normally, NF κ B is bound in the cytosol to its inhibitor κ B (I κ B) to prevent activation of NF κ B. Inflammatory signals can cause phosphorylation of I κ B, thereby releasing and activating NF κ B, followed by translocation of NF κ B to the nucleus and activation of genes involved in the pro-inflammatory response, such as TNF α and matrix metalloproteinases⁴. To further understand in humans the *in vivo* (anti-inflammatory) effects of rosiglitazone treatment at the molecular level, it would be advantageous that Peripheral Blood Mononuclear Cells (PBMCs) could be used. These cells are readily accessible in humans compared to the relatively inaccessible target tissues of PPAR γ ligands, namely adipose tissue and liver⁵. It has already been demonstrated that monocytes respond to PPAR γ ligands by reducing the expression of inflammatory cytokines *ex vivo*, such as TNF α , IL-6 and IL1b⁶. In addition, Mohanty *et al.*³ have shown that rosiglitazone inhibits the binding of NF κ B to DNA in the nucleus of PBMCs from obese and obese diabetic patients. This was accompanied by an anti-inflammatory effect, as indicated by reduced plasma hsCRP and MCP-1 concentrations³. However, it is not known if rosiglitazone's anti-inflammatory effect changes the transcription of NF κ B related genes in PBMCs *in vivo*. Therefore, the first aim of our study was to examine if anti-inflammatory properties of rosiglitazone are reflected in changes in expression of genes involved in the NF κ B signaling pathway in PBMCs. Furthermore, the role of an increased insulin concentration in low-grade systemic inflammation, as often seen in type 2 diabetic patients, is not completely understood⁷. Therefore, the second aim of this study was to examine plasma inflammatory markers in the fasted (hyperglycaemic) state and during hyperinsulinemia using a hyperinsulinemic-euglycemic clamp.

2. Methods

2.1 Subjects

Twelve middle-aged obese men with well-controlled type 2 diabetes mellitus participated in this study. Due to extreme changes in serum cholesterol concentrations and gene expression profiles, one subject was excluded from the statistical analyses. The characteristics of the 11 remaining (age, 62 ± 5 y; body mass index (BMI), 31 ± 3 kg/m²) type 2 diabetic subjects and 10 BMI and age matched normoglycemic controls (age, 57 ± 8 y; BMI, 30 ± 4 kg/m²) are shown in

table 1. Type 2 diabetes mellitus was diagnosed at least 1 year before the study, and most patients were treated with sulphonylurea (n=3) or metformin (n=5) or both (n=1) as anti-diabetic medication. Control subjects had normal glucose homeostasis, as determined with a standard oral glucose tolerance test, and had no family history of diabetes. The Medical Ethical Review Board of Maastricht University had approved the study and written, informed consent was obtained from all volunteers.

2.2 Study design

Details of this study have been described previously^{8,9}. In short, diabetic patients stopped any anti-diabetic medication 14 days before the start of the study. Subjects were asked not to participate in (exhaustive) physical activity the last three days preceeding the measurements, and to consume a diet according to the Dutch guidelines for a healthy diet. After a baseline hyperinsulinemic-euglycemic clamp, diabetic patients were treated with rosiglitazone (Avandia®, GlaxoSmithKline, Zeist, the Netherlands), 8 mg/day (2 x 4 mg) for 8 weeks. After rosiglitazone treatment, subjects underwent a second clamp. Blood samples were drawn before and after the clamp, thereby providing fasting and insulin-stimulated blood samples before and after rosiglitazone treatment. The non-diabetic patients served as baseline controls to the diabetic patients. They also underwent a hyperinsulinemic-euglycemic clamp, but were not treated with rosiglitazone.

2.3 Hyperinsulinemic-euglycemic clamp

After an overnight fast, subjects came to the laboratory at 8 AM. A cannula was inserted into each antecubital vein for the infusion of tracer, insulin and glucose. A third cannula was inserted retrogradely into a superficial dorsal hand vein for arterialized blood sampling. After taking fasting blood samples, a primed constant infusion of [6,6]-²H₂-glucose was initiated (0.04 mg/kg·min) for 300 minutes. At t=120 min, a 3-hour primed constant infusion of insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was started (40 mU/m²·min), and glucose was clamped by a variable co-infusion of 20% glucose with tracer added. Blood samples taken at t=0 min and t=300 min were used for further analysis.

2.4 Plasma measurements

EDTA plasma was obtained by 10 minutes of centrifugation at 3000 rpm immediately after sampling, and stored at -80°C for later analysis. Concentrations of total cholesterol (ABX Diagnostics, Montpelier, France), HDL cholesterol (precipitation method; Roche Diagnostics Corporation, Indianapolis, IN), and triglycerides corrected for free glycerol (Sigma-Aldrich Chemie, Steinheim, Germany) were analysed enzymatically. Serum LDL cholesterol concentrations were calculated by using the formula of Friedewald *et al.*¹⁰. Insulin concentrations were measured using a RIA (Linco Research, St. Charles, MO, USA). Free fatty acids (FFA) were determined using the Wako Nefa C test kit (Wako Chemicals,

Neuss, Germany) and plasma glucose was determined by using the hexokinase method (LaRoche, Basel, Switzerland). The glucose infusion rate (GIR) was used as a measure of insulin sensitivity. For the inflammation markers, high sensitive CRP (hsCRP) was measured on Cobas Mira with a commercial available kit (Kamiya Biomedical Company, Seattle, WA, USA) and TNF α , IL6 and MCP1 were measured with an ELISA kit from R&D systems. All samples from one subject were analysed in the same analytical run. Samples were corrected for plasma albumin concentrations to correct for changes in blood volume due to fluid infusion during the hyperinsulinemic-euglycemic clamp¹¹.

2.5 *PBMC isolation and total RNA isolation*

PBMCs were isolated from an EDTA anti-coagulated peripheral fasted blood sample of diabetic patients only, directly after blood sampling through gradient centrifugation using lymphoprep (Nycomed, Oslo, Norway) according to the instructions from the manufacturer. The obtained PBMCs were immediately lysed and homogenised in 1.5 ml Trizol (Invitrogen Life Technologies, Breda, The Netherlands) for RNA stabilisation and subsequent RNA isolation according a standardized protocol as described by the manufacturer. Next, RNA was purified using the RNeasy mini kit (Qiagen Benelux B.V., Venlo, the Netherlands) followed by dissolving the RNA in RNase and DNase free water (Invitrogen Life Technologies, Breda, The Netherlands). RNA purity was measured on the NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA), and considered suitable for further processing at 260/280 and 260/230 ratios of >1.7 . Integrity was evaluated using the BioAnalyzer (Agilent, Palo Alto CA, USA) and considered to be intact with an RNA integrity number > 7.0 .

2.6 *Real time PCR*

cDNA was synthesised using the ReactionReady™ First Strand cDNA Synthesis Kit (SuperArray Bioscience corporation, Frederick, MD, USA). The total amount of RNA used in the reaction varied from 800 ng to 1 μ g. Equal amounts of RNA were used for samples before and after treatment of the same subject.

Gene expression of NF κ B pathway focussed genes was analysed using NF κ B RT²Profiler PCR arrays, according to the manufacturer's instructions (SuperArray Bioscience Corporation, Frederick, MD, USA). The PCR array consisted of a 96-well plate including primer sets of 84 NF κ B related genes (see **additional file 1**), plus 5 housekeeping genes and 2 negative controls. The primer sets were optimised for real time detection using RT²Real Time™ SYBR Green PCR master mix (SuperArray Bioscience Corporation, Frederick, MD, USA). In addition, the relative expression levels of PPAR γ , CD36 and LPL, were also determined with real time PCR, using assays-on-demand and Taqman Universal Mastermix (Applied Biosystems, Foster City, CA, USA). All gene expression analyses were performed on the ABI PISM 7000 system (Applied Biosystems, Foster City, CA, USA).

2.7 *Data analysis of real time PCR data*

The No Template Control tested for DNA contamination in the PCR system and the No Reverse Transcription Control tested for contamination of the original RNA with genomic DNA. Those threshold cycles were above 35, which indicates that there was no contamination. The average C_t values of the housekeeping genes, β -actin and 18srRNA, were used for normalisation of each individual sample. To express the difference in expression before and after rosiglitazone treatment in diabetic patients, the $\Delta\Delta C_t$ value was calculated for each gene according to the comparative C_t method, and was used for statistical analysis.

2.8 *Statistics*

Fasting plasma parameters are presented as mean $\pm$ SEM. Gene expression levels were not normally distributed and are therefore expressed as median $\Delta\Delta C_t$ values together with corresponding ranges. An independent Student's t-test was used to compare diabetic patients before or after rosiglitazone with control subjects. Results of plasma parameters before and after rosiglitazone treatment in type 2 diabetic patients were analysed using a paired Student's t-test. The effect of rosiglitazone on gene expression levels in type 2 diabetic patients was analysed with the non-parametric Wilcoxon signed rank test. Changes (%) in plasma parameters during the hyperinsulinemic-euglycemic clamp (T0 vs. T300) were tested with a one sample t-test. In addition, using the Student's t-test, the changes in plasma parameters induced by the hyperinsulinemic-euglycemic clamp before rosiglitazone treatment were compared to the clamp-induced changes after rosiglitazone treatment.

All statistical analyses were performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1. BMI and plasma parameters of type 2 diabetic patients before and after rosiglitazone treatment and of non-diabetic controls.

	Diabetic patients (n=11)						Controls (n=10)		
	Before treatment			After treatment					
BMI (kg/m ²)	30	±	1.0	31	±	1.0	31	±	1.3
Glucose (mmol/L)	9.2	±	0.5 ^a	8.1	±	0.5 ^{b,c}	5.8	±	0.1
Insulin (mU/L)	24.1	±	3.1 ^a	17.2	±	1.7 ^{b,c}	12.3	±	0.9
GIR (μmol/kg/min)	12.8	±	1.3 ^a	20.6	±	1.7 ^b	24.6	±	2.4
Total cholesterol (mmol/L)	5.4	±	0.3	5.7	±	0.4	5.2	±	0.3
LDL cholesterol (mmol/L)	3.5	±	0.2	3.8	±	0.4	3.4	±	0.3
HDL cholesterol (mmol/L)	0.93	±	0.1	1.10 ^b	±	0.1 ^b	1.14	±	0.1
TCH/HDL ratio	6.9	±	1.1	5.5	±	1.0	5.5	±	1.1
Triglycerides (mmol/L)	2.0	±	0.3	1.5 ^b	±	0.2 ^b	1.5	±	0.4
FFA (μmol/L)	509	±	47	400	±	37 ^b	513	±	45.6

^a Significantly different (P<0.05) between diabetic patients and non-diabetic controls

^b Significant effect (P<0.05) of rosiglitazone treatment in diabetic patients

^c Significantly different (P<0.05) between diabetic patients after rosiglitazone treatment and non-diabetic controls

3. Results

3.1 Body weight

There was no difference in body weight between diabetic patients and the BMI-matched controls (94.3 ± 4.1 kg and 92.7 ± 4.1 kg, respectively ($p=0.778$)). Rosiglitazone treatment resulted in a slight, but non-significant increase in body weight during the 8 weeks follow-up period in the diabetic patients (1.3 ± 1.0 kg, $p=0.21$).

3.2 Plasma glucose and insulin concentrations and insulin sensitivity

As expected, diabetic patients were less insulin-sensitive and had higher fasting plasma glucose and insulin concentrations compared with non-diabetic controls (**table 1**). Fasting glucose and insulin were significantly decreased in the diabetic patients after rosiglitazone treatment (-1.0 ± 0.5 mmol/L, $p=0.044$) and (-6.9 ± 2.3 mU/L, $p=0.013$, respectively), while insulin sensitivity increased (change in GIR: $+7.8 \pm 1.9$ μmol/kg/min, $p=0.002$) (**table 1**).

3.3 Plasma lipid concentrations

As shown in **table 1**, there were no significant differences in plasma total cholesterol, LDL cholesterol, HDL cholesterol, TCH/HDL ratio, triglycerides and free fatty acids (FFA) between diabetic patients and non-diabetic controls. Fasting HDL cholesterol increased significantly ($+0.21 \pm 0.07 \mu\text{mol/L}$, $p=0.013$), while triglyceride and FFA concentrations decreased ($-0.48 \pm 0.21 \mu\text{mol/L}$, $p=0.042$ and $-109 \pm 38 \text{ mmol/L}$, $p=0.018$ respectively) upon rosiglitazone treatment in diabetic patients. Total cholesterol and LDL cholesterol concentrations were not significantly affected by rosiglitazone treatment ($+0.30 \pm 0.20 \text{ mmol/L}$, $p=0.152$ and $+0.30 \pm 0.20 \text{ mmol/L}$, $p=0.160$, respectively). The TCH/HDL ratio tended to improve after treatment (-1.3 ± 0.7), but this did not reach statistical significance ($p=0.09$).

3.4 Plasma hsCRP, $\text{TNF}\alpha$, IL6 and MCP1 concentrations

As shown in **figure 1**, the basal plasma concentration of hsCRP in the diabetic patients was $2.2 \pm 0.4 \text{ mg/L}$, which was significantly higher than that of the non-diabetic controls ($1.0 \pm 0.2 \text{ mg/L}$, $p=0.027$). There were no differences in $\text{TNF}\alpha$, IL6 and MCP1 concentrations between diabetic patients and control subjects. As a result of rosiglitazone treatment, basal hsCRP and MCP1 concentrations significantly decreased ($-1.1 \pm 0.3 \text{ mg/L}$, $p=0.003$ and $-9.5 \pm 5.3 \text{ pg/mL}$, $p=0.043$, respectively) and, paradoxically, $\text{TNF}\alpha$ concentrations increased ($+0.22 \pm 0.1 \text{ pg/mL}$, $p=0.037$) in type 2 diabetic patients. After rosiglitazone treatment, hsCRP ($1.0 \pm 0.16 \text{ mg/L}$) and MCP1 concentrations ($132 \pm 7 \text{ pg/mL}$) in diabetic patients were comparable with those measured in non-diabetic controls.

Figure 1. Concentrations of plasma inflammation markers in controls and in diabetics before and after rosiglitazone treatment.

Baseline concentrations of plasma inflammatory markers, A) hsCRP, B) $\text{TNF}\alpha$, C) IL6 and D) MCP1, in type 2 diabetic and control subjects and levels after rosiglitazone treatment in type 2 diabetic patients. * = p -value < 0.05

3.5 Expression of PPAR γ , PPAR γ responsive genes and NF κ B related genes

Of the 84 genes measured, 75 were detectable in PBMCs and only the expression of genes encoding for IFN γ (interferon gamma), IL1R1 (interleukin 1 receptor 1), RELB (V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)) and SLC20A1 (solute carrier family 20 (phosphate transporter) member 1) changed significantly in the diabetic patients upon rosiglitazone treatment (**table 2**).

The expression level of PPAR γ itself was not changed by its ligand rosiglitazone. Also expression levels of CD36 (thrombospondin receptor) and LPL (lipoprotein lipase), which are known PPAR γ target genes¹²⁻¹⁴, did not change (**table 2**).

Table 2. Effect of rosiglitazone treatment on expression levels (ΔC_t) of PPAR γ , PPAR γ responsive genes and NF κ B related genes in type 2 diabetic patients¹.

Gene	$\Delta\Delta C_t$ (after - before treatment)	p -value ³
PPAR γ	0.31 (-0.70 - 1.29)	0.213
CD36	0.37 (-0.93 - 0.90)	0.248
LPL	-0.39 (-0.90 - 1.26)	0.248
INF γ	-0.53 (-3.04 - 0.62)	0.045
IL1R1	0.37 (-1.16 - 1.51)	0.041
RELB	0.31 (-0.23 - 2.27)	0.016
SLC20A1	0.41 (-1.03 - 1.93)	0.033

¹ The expression of 84 genes related to NF κ B was profiles using the RT²Profiler™ PCR Array; n=11

² Values are reported as $\Delta\Delta C_t$ values and presented as medians with ranges. A negative $\Delta\Delta C_t$ value corresponds with an upregulation and a positive $\Delta\Delta C_t$ value with a downregulation of the gene after rosiglitazone treatment.

³ Gene expression levels (ΔC_t values) were analysed with the non-parametric Wilcoxon signed rank test. Differences were considered significant at a p -value < 0.05

Table 3. Relative changes in plasma parameters upon insulin-stimulation during the hyperinsulinemic-euglycemic clamp.

	Diabetic patients (n=11)						Controls (n=10)		
	Before treatment			After treatment					
Albumin (%)	-3.4	±	0.9 [#]	-3.1	±	1.1 [#]	-6.0	±	0.8 [#]
Total cholesterol (%)	0.4	±	0.8	-1.7	±	1.4	0.4	±	0.7
LDL cholesterol (%)	2.5	±	2.1	-0.5	±	1.3	2.3	±	1.1
HDL cholesterol (%)	1.3	±	2.7	0.8	±	1.6	2.7	±	1.3
Triglycerides (%)	-5.2	±	6.4	-14.3	±	5.5 ^{a,c}	-16.7	±	4.5 ^a
FFA (%)	-58.9	±	3.4 ^{a,b}	-65.0	±	4.1 ^{a,c}	-80.1	±	2.6 ^a
hsCRP (%)	-4.7	±	2.7	-4.9	±	2.4	5.5	±	4.5
TNF α (%)	12.0	±	6.6	-9.1	±	7.9 ^c	12.1	±	14.2
IL6 (%)	13.7	±	6.7	16.4	±	7.9	23.5	±	9.0 ^a
MCP1 (%)	-9.1	±	1.8 ^a	-3.7	±	4.0	-11.1	±	4.1 ^a

[#] Plasma albumin changed ($p < 0.05$) during the clamp because of increase in blood volume due to fluid infusion. Therefore, all plasma parameters are corrected for changes in plasma albumin concentration and presented as mean $\pm$ SEM

^a Significant change ($P < 0.05$) in response to insulin

^b Significant difference between type 2 diabetic patients and BMI-matched controls in response to insulin

^c Significant change ($P < 0.05$) effect of rosiglitazone treatment on the response to insulin in type 2 diabetic patients

3.6 Changes in plasma parameters during the clamp

Before rosiglitazone treatment, plasma albumin concentrations were 38.1 ± 0.6 g/L before the clamp and 36.8 ± 0.5 g/L after the clamp ($p = 0.005$). These concentrations were comparable after rosiglitazone treatment. For the control subjects, these values were before and after the clamp respectively 38.7 ± 0.6 g/L and 36.4 ± 0.7 g/L ($p < 0.001$). The observed reductions in plasma albumin concentration indicate that due to the glucose infusion, the plasma volume had changed during the clamp. Therefore, levels of the plasma parameters were related to those of albumin. It is known that plasma albumin concentrations are stable during the day¹¹.

Before treatment, serum total, LDL, and HDL cholesterol levels did not change in response to insulin during the hyperinsulinemic-euglycemic clamp in both type 2 diabetic patients and control subjects (table 3). Triglycerides levels decreased

in control subjects ($-16.7 \pm 4.5\%$, $p=0.007$) and also in diabetic patients after rosiglitazone treatment ($-14.3 \pm 5.5\%$, $p=0.027$). FFA levels decreased significantly in type 2 diabetic patients ($-58.9 \pm 3.4\%$, $p<0.001$) in response to insulin. In control subjects the reduction in FFA was even more pronounced ($-80.1 \pm 2.6\%$, $p<0.001$). Rosiglitazone improved the insulin-induced decrease in FFA (from $-58.9 \pm 3.4\%$ to $-65.0 \pm 4.1\%$, $p=0.045$).

Insulin significantly decreased MCP1 levels in type 2 diabetic patients before rosiglitazone treatment ($-9.1 \pm 1.8\%$, $p=0.001$) and control subjects ($-11.1 \pm 4.1\%$, $p=0.023$) (table 3). Control subjects also showed a significant increase in IL6 levels ($23.5 \pm 9.0\%$, $p=0.028$). Changes in $\text{TNF}\alpha$ levels upon insulin stimulation differed significantly before and after rosiglitazone treatment in diabetic patients. Levels of $\text{TNF}\alpha$ increased during the clamp before rosiglitazone treatment ($+12.0 \pm 6.6\%$), but decreased after rosiglitazone treatment ($-9.1 \pm 7.9\%$, $p=0.006$).

4. Discussion

In this study, 8 weeks of rosiglitazone treatment (2x4 mg/d) not only improved insulin sensitivity and plasma lipids, but also lowered fasting plasma concentrations of hsCRP and MCP1. After rosiglitazone, fasting concentrations of hsCRP and MCP1 in diabetic patients were even comparable to those of non-diabetic controls. These anti-inflammatory changes were not reflected in the expression of $\text{NF}\kappa\text{B}$ -related genes in PBMCs.

Reductions in fasting triglyceride concentrations have been reported for other TZDs, such as pioglitazone, troglitazone and darglitazone. We have now shown that these results can be extended to rosiglitazone. Furthermore, FFA concentrations were also reduced after rosiglitazone treatment. These reductions may be explained by an increased clearance of TG and FFA, as shown by Dhindsa *et al.*¹⁵. Rosiglitazone treatment further significantly increased serum HDL cholesterol concentrations and total cholesterol and LDL cholesterol tended to increase. Generally, the effects of rosiglitazone on serum lipid concentrations were comparable to those of other TZDs¹⁶.

Rosiglitazone treatment in type 2 diabetic patients decreased hsCRP and MCP1 concentrations, indicating a reduced inflammatory state. Mohanty *et al.* also observed a significant reduction in MCP1 and hsCRP concentrations after 6 weeks of rosiglitazone treatment (4 mg/d) in non-diabetic obese subjects and obese diabetic patients³. In accordance with previous studies^{2, 17, 18}, we did not observe an effect of rosiglitazone on plasma IL6 concentrations. The increase we observed in $\text{TNF}\alpha$ concentrations is unexpected, although studies on the effects of TZDs on plasma $\text{TNF}\alpha$ concentrations are inconsistent. Some studies have shown that plasma concentrations of this cytokine are increased in subjects with insulin resistance or type 2 diabetes^{19, 20}. A number of other studies, however, showed reduced $\text{TNF}\alpha$ concentrations^{21, 22} after rosiglitazone treatment in type 2 diabetic patients, whereas others found no effect¹⁸. In line with our observations, Goldstein *et al.*²³ showed that $\text{TNF}\alpha$ concentrations were significantly increased

when rosiglitazone was added to metformin treatment in type 2 diabetic patients. Due to these inconsistent results, a clear explanation is lacking.

Despite the rosiglitazone-induced reductions in fasting plasma CRP and MCP1, which are under control of NF κ B^{24, 25}, expression of NF κ B-related genes in PBMCs hardly changed upon rosiglitazone treatment. Of the 75 detectable NF κ B-related genes in PBMCs, only 4 genes were significantly altered. The gene encoding for interferon gamma (INF γ) was significantly upregulated, whereas IL1 β , RELB and SLC20A1 were significantly downregulated by rosiglitazone treatment. These changes would indicate an inhibition of the NF κ B signalling cascade^{4, 26-28}. The significant reduction in MCP1 and hsCRP concentrations after 6 weeks of rosiglitazone treatment (4 mg/d) in non-diabetic obese subjects and obese diabetic patients as described by Mohanty *et al.*, was accompanied by a significant fall in intranuclear NF κ B levels in PBMCs³. This suggests a direct interference of PPAR γ in the binding of NF κ B to the promotor regions of proinflammatory genes. However, these reduced intranuclear NF κ B levels were not accompanied by a changed expression of the NF κ B-related genes I κ B and p65 (REL A) after rosiglitazone treatment^{3, 29}. For troglitazone, another PPAR γ agonist, it has also been demonstrated that intranuclear and cellular levels of NF κ B were decreased in mononuclear cells of obese subjects³⁰. Unfortunately, these studies did not examine if the fall in intranuclear NF κ B levels was accompanied by a reduced expression of inflammatory NF κ B target genes. In our study, the lack of effect on NF κ B related genes by rosiglitazone in PBMCs suggests that PBMCs are insensitive to rosiglitazone.

To further substantiate whether PBMCs were non-responsive to rosiglitazone treatment, we evaluated if rosiglitazone influenced gene expression of specific PPAR γ dependent genes in PBMCs *in vivo*, i.e., PPAR γ itself, CD36 and lipoprotein lipase (LPL). *In vitro* and *ex vivo* studies have shown that expression of CD36^{31, 32} and LPL³³ in macrophages increased after activation of PPAR γ . In our study, however, the *in vivo* expression of PPAR γ , CD36 and LPL in PBMCs was not altered by rosiglitazone treatment, which suggests that PBMCs are non-responsive to rosiglitazone. In contrast to most other studies, we used PBMCs, a mixed population of white blood cells, and studied the *in vivo* effects. Most studies that did observe anti-inflammatory effects of rosiglitazone focussed on *in vitro* effects in a specific subpopulation of blood cells, namely monocytes or macrophages. These studies showed an increased expression of PPAR γ and PPAR γ responsive genes when these cells were cultured *ex vivo* or *in vitro*, and/or were given extra cytokine stimulation^{31, 34, 35}. *In vivo*, Bouhlef *et al.*³⁴ showed a significant upregulation of PPAR γ expression in PBMCs of subjects after 2 months of 45 mg/day pioglitazone (TZD) treatment in patients with severe atherosclerosis, although expression of the PPAR γ dependent genes, CD136 and mannose receptor, did not change. Combining these finding with our results suggests that gene expression profiles in PBMCs are not suitable to use as a biomarker to study *in vivo* the effects of rosiglitazone intervention at a molecular level. Whether specific subpopulations of cells within PBMCs are suitable, warrant further investigation. In this respect,

study of monocytes, preferably isolated from blood via for example cell sorter techniques, warrants attention as especially the monocytes play an important role in the inflammation process and insulin resistance. Arkan *et al.*³⁶, for example, showed that deletion of IKK β , required for the activation of NF κ B, in myeloid precursor cells protected animals from diet-induced obesity-related insulin resistance.

To investigate the acute effect of insulin on the low-grade pro-inflammatory profile in type 2 diabetic patients at constant glucose concentrations, plasma inflammation parameters were analysed before and after the hyperinsulinemic-euglycemic clamp. Results, however, were not conclusive. MCP1 level decreased in both diabetic and control subjects, IL6 level increased only in control subjects, while hsCRP and TNF α levels did not significantly change. The reduced MCP1 level after insulin stimulation can, however, also be an indirect effect of insulin and explained for example by the insulin-mediated reduction in FFA³⁷. The causal role of IL6 in the development of insulin resistance and type 2 diabetes is not clear. Infusion with a physiological concentration of IL6 in humans increased the uptake of glucose in subcutaneous adipose tissue *in vivo*³⁸. On the other hand, IL6 levels are increased in type 2 diabetic subjects. Like in our study, Krogh-Madsen *et al.* found an increase in plasma IL6 concentrations during a hyperinsulinemic-euglycemic clamp in healthy human males³⁹. This increase was accompanied by an elevated insulin-stimulated increase in IL6 gene expression in adipose tissue. Also, they found no change in plasma concentrations of TNF α , which expression was increased in adipose tissue and reduced in muscle tissue³⁹. The reason that we did not find a reduction in hsCRP concentration may have been due to the relative short time of insulin infusion in our study, as previous studies demonstrated decreased hsCRP concentrations after 10 or more hours of insulin infusion⁴⁰⁻⁴². It also needs to be mentioned that during the hyperinsulinemic-euglycemic clamp, not only insulin infusion can exert biological effects, but also the amount of glucose infused to maintain euglycemia⁴³. However, Dandona *et al.* have demonstrated an acute anti-inflammatory effect of insulin infusion at a physiological concentration, infusing insulin (2.0-2.5 IU/h) - dextrose(5%) and in obese non-diabetic subjects, while maintaining glucose levels as close to basal levels as possible⁴¹. This anti-inflammatory effect was reflected by reduced intranuclear levels of NF κ B, increased I κ B, decreased ROS generation and decreased p47phox subunit in PBMCs, decreased plasma intracellular adhesion molecule 1, plasminogen inhibitor 1 and MCP1 concentrations⁴¹. Infusion with only dextrose or saline had no effects on these parameters⁴¹. Taken together, a supra-physiological increase in insulin concentration does not per se contribute to the disturbances in inflammatory markers in type 2 diabetic patients.

In conclusion, 8 weeks of rosiglitazone treatment (2x4 mg/d) resulted in improved insulin sensitivity and lipid profile and reduced concentrations of plasma inflammatory markers (MCP1 and hsCRP) in type 2 diabetic patients. Furthermore, plasma inflammatory parameters did not change consistently during the clamp in both diabetic and control patients, which does not suggest that high insulin levels

contribute to the proinflammatory state in type 2 diabetic patients. Finally, the anti-inflammatory effect of rosiglitazone is not reflected by changes in NF κ B and PPAR γ target genes in PBMCs *in vivo*.

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CHAPTER 4

Comparison of the effects of n-3 long chain polyunsaturated fatty acids and fenofibrate on markers of inflammation and vascular function, and on the serum lipoprotein profile in overweight and obese subjects

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Abstract

The aim of this study was to compare the effects of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) from fish oil, with those of fenofibrate, on markers of inflammation and vascular function, and on the serum lipoprotein profile in overweight and obese subjects. Twenty overweight and obese subjects participated in a randomized, double-blind, placebo-controlled intervention trial and received 3.7 g/d n-3 fatty acids (providing 1.7 g/d EPA and 1.2 g/d DHA), 200 mg fenofibrate or placebo treatment for 6 weeks separated by a 2-weeks wash-out period. Fish oil and fenofibrate treatment reduced triglyceride (-0.61 ± 0.81 mmol/L, $P < 0.001$, and -0.34 ± 0.85 mmol/L, $P = 0.048$, respectively) and increased HDL cholesterol concentrations (0.13 ± 0.21 mmol/L, $P = 0.013$, and 0.10 ± 0.18 mmol/L, $P = 0.076$), as reflected by a decrease of large very VLDL particles and increases of large HDL particles and medium size HDL particles. Fish oil increased serum LDL cholesterol concentrations (0.34 ± 0.59 mmol/L, $P = 0.013$). Fenofibrate reduced concentrations of soluble endothelial selectin (sE-selectin) (-4.1 ± 7.5 ng/mL, $P = 0.032$), but increased those of macrophage chemoattractant protein 1 (MCP1) (28 ± 55 ng/mL, $P = 0.034$). Fish oil had no effects on these markers. Although n-3 LCPUFA and fenofibrate can both activate PPAR α , they have differential effects on cardiovascular risk markers. In overweight and obese subjects fenofibrate (200 mg/d) or n-3 LCPUFA (3.7 g/d) treatment for 6 weeks did not improve markers for low-grade systemic inflammation, while fenofibrate had more profound effects on plasma lipids and markers for vascular activity compared to fish oil.

1. Introduction

Drugs of the fibrate class, such as fenofibrate, are potent activators of Peroxisome Proliferator Activated Receptor α (PPAR α)¹. These lipid-lowering drugs effectively reduce triglyceride, moderately reduce low density lipoprotein (LDL) cholesterol, and elevate high density lipoprotein (HDL) cholesterol². Furthermore, fibrates may exert anti-inflammatory effects and improve vascular function³. Therefore, targeting PPAR α can be an effective way to improve features belonging to the metabolic syndrome and to reduce cardiovascular risk. As PPARs can be seen as lipid sensors, dietary n-3 fatty acids deserve attention in this respect. Especially the marine n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) preferentially bind to and activate PPAR α ¹. However, these n-3 LCPUFA can also activate PPAR γ and PPAR δ , two other PPAR isoforms¹. As fibrates, dietary n-3 LCPUFA have potent hypotriglyceridemic effects and can increase HDL cholesterol⁴. Furthermore, the suggested beneficial effects on inflammation and endothelial function may further contribute to a reduction in cardiovascular risk. Stalenhoef *et al.* have now compared in hypertriglyceridemic subjects gemfibrozil with n-3 LCPUFA and showed that both treatments had favorable effects on serum lipid concentrations and lipoprotein particle heterogeneity⁵. However, in that study markers reflecting low-grade systemic inflammation and endothelial function were not examined.

To establish the relevance of a dietary component as a subtle, PPAR agonist with that of a strong, synthetic PPAR α agonist, we decided to compare side-by-side the effects of n-3 LCPUFA with those of fenofibrate on inflammatory parameters, vascular function, and the serum lipoprotein profile in overweight and obese subjects, who are at increased risk to have or to develop the metabolic syndrome.

2. Methods

2.1 Subjects

Caucasian subjects with a BMI of at least 27 kg/m² were recruited between the end of March and the end of August of 2007 via posters in the university and hospital buildings, and via advertisements in local newspapers. Subjects came to the university for a screening visit. On this visit, fasting blood was sampled for analyses of serum lipids and lipoproteins. In addition, height and body weight were determined. Furthermore, subjects had to complete a medical and general questionnaire. Exclusion criteria were BMI below 27 kg/m², impairment of kidney (creatinine > 150 mmol/L) and liver function (ALAT, ASAT, ALP, GGT or total bilirubine > 2 times upper limit of normal), serum total cholesterol above 8 mmol/L, serum triglycerides above 4 mmol/L, taking medication that could influence the study outcome or could interfere with fenofibrate treatment, use of fish oil supplements, consumption of plant sterol or stanol-enriched food products, having donated blood within 1 month prior to the start of the

study, having a diagnosis of any long-term medical condition (e.g. diabetes, cardiovascular diseases, epilepsy) or experiencing strong symptoms of allergy. Subjects received oral and written information about the nature and risk of the experimental procedures before their written informed consent before the start of the study. The study was approved by the Medical Ethical Committee of Maastricht University.

After the screening of 34 subjects, 26 subjects met all our inclusion criteria and started the study. After inclusion, 6 subjects dropped out (1 man underwent surgery for an aneurysm, 1 woman had complained about vapours during the placebo period, 1 man and 1 woman did not regularly attend appointments and were excluded, 1 man had a work-related reason, and 1 man had personal reasons). Thus, ten men and ten women completed the trial. Baseline characteristics are presented in **table 1**.

Table 1. Baseline characteristics of the participants.

	All (n=20)			Females (n=10)			Males (n=10)		
Age (years)	52	±	12	51	±	14	54	±	10
BMI (kg/m ²)	33	±	5	34	±	5	31	±	5
Waist circumference (cm)	117	±	12	116	±	11	118	±	13
Systolic blood pressure (mmHg)	131	±	14	122	±	9	141	±	10
Diastolic blood pressure (mmHg)	91	±	8	85	±	7	96	±	5
Total cholesterol (mmol/L)	6.23	±	1.18	5.89	±	1.45	6.59	±	0.78
LDL cholesterol (mmol/L)	3.97	±	1.09	3.54	±	1.23	4.41	±	0.75
HDL cholesterol (mmol/L)	1.52	±	0.44	1.67	±	0.46	1.38	±	0.39
Triglycerides (mmol/L)	1.63	±	0.59	1.51	±	0.52	1.76	±	0.65
Glucose (mmol/L)	5.34	±	0.73	5.22	±	0.54	5.45	±	0.88
Criteria metabolic syndrome (n) ²	2.2	±	1.0	1.6	±	0.7	2.7	±	1.1
Diagnosis metabolic syndrome (n) ²	7			1			6		

¹ Values are expressed as mean ± SD

² According to the National Cholesterol Education Program Adult Treatment Panel III guidelines

2.2 Study design

The study had a randomized, double-blind, placebo-controlled, crossover design. Each subject enrolled in random order in a fish oil, a fenofibrate and a placebo

period for 6 weeks with a wash-out period of at least 2 weeks between the intervention periods. During the fish oil intervention, subjects had to consume daily 8 fish oil capsules (Marinol C-38™, Lipid Nutrition, Wormerveer, the Netherlands), providing approximately 3.7 g/d n-3 LCPUFA (1.7 g/d EPA and 1.2 g/d DHA,) and 2 capsules placebo-matching fenofibrate (200 mg/d cellulose). During the fenofibrate period, subjects consumed 2 capsules providing 200 mg/d micronized fenofibrate (Lipanthyl®, Fournier Laboratories, Dijon, France) and 8 placebo-matching fish oil capsules (containing 80% High Oleic Sunflower Oil (HOSO)). During the placebo period, subjects received 8 HOSO capsules and 2 cellulose capsules. Subjects had to ingest half of the capsules before breakfast and the other half before dinner with a glass of water. Subjects were restricted in their fish consumption to a maximum of one portion a week. During the study, subjects recorded any symptom of illness, visits to physician, medication used, alcohol consumption, and any deviations from the protocol in diaries. Body weight was recorded at weeks 0, 5 and 6 of each intervention period and blood pressure was monitored using a sphygmomanometer (Omron M7, CEMEX Medische Techniek BV, Nieuwegein, the Netherlands). At the end of each intervention period, energy and nutrient intakes of the previous 4 weeks were estimated using a food frequency questionnaire (FFQ)⁶.

2.3 Blood sampling and analyses

2.3.1 Blood sampling

In weeks 5 and 6 of each intervention period, subjects arrived in the morning after an overnight fast and after abstinence from drinking alcohol the preceding day. Venous blood was sampled in BD vacutainer® tubes (Becton Dickinson Company, NJ, USA). Serum was obtained by clotting the blood for 30 minutes, followed by 30 min centrifugation at 2000×g. EDTA, NaF and heparin plasma were obtained by centrifugation at 2000×g for 30 minutes at 4°C, directly after sampling. Serum and plasma aliquots were snap frozen and stored at -80°C until analysis.

2.3.2 Clinical safety parameters

Serum concentrations of markers of liver and kidney function (total bilirubin, aspartate aminotransferase (ASAT), alanine-aminotransferase (ALAT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GT), ureum, and creatinine) from week 6 of each intervention period were determined at the department of Clinical Chemistry, University Hospital Maastricht (Beckman Synchron CX7 Clinical systems, Beckman).

2.3.3 Plasma lipids, lipoproteins and glucose

Plasma EDTA samples from weeks 5 and 6 were analysed separately for concentrations of serum total cholesterol (ABX Diagnostics, Montpellier,

France), HDL cholesterol (precipitation method; Roche Diagnostics Corporation, Indianapolis, IN), and triglycerides corrected for free glycerol (Sigma-Aldrich Chemie, Steinheim, Germany). Serum LDL cholesterol concentrations were calculated with the formula of Friedewald *et al*⁷. After analysis, values of weeks 5 and 6 were averaged. The free EPA and DHA content in plasma as a compliance marker, was determined with LC-MS methodology (TNO, Zeist, the Netherlands) as described⁸ in heparin plasma of week 6 from each period. The plasma lipoprofile (number and size of lipoprotein particles) was analysed by NMR (NMR LipoProfile test, Liposcience Inc., Raleigh, NC, USA) in a pooled sample from weeks 5 and 6 of each treatment period. NaF plasma samples from weeks 5 and 6 were analysed for free fatty acids (FFA) with the Wako Nefa C test kit (Wako Chemicals, Neuss, Germany) and plasma glucose with the hexokinase method (LaRoche, Basel, Switzerland), and values were averaged.

2.3.4 *Markers of inflammation and vascular activity*

Plasma EDTA samples from weeks 5 and 6 of each intervention period were pooled prior to the analysis of plasma markers of inflammation and vascular activity. High sensitive CRP (hsCRP) was measured with a immunoturbidimetric assay using commercially available kit (Kamiya Biomedical Company, Seattle, WA, USA). The MS2400 Human Vascular Injury II Assay (Meso Scale Discovery, MD, USA) was used for analysis of soluble Vascular Cell Adhesion Molecule-1 (sVCAM1) and soluble Intracellular Adhesion Molecule-1 (sICAM1). Tumor Necrosis Factor α Receptors (TNFR) 1 and 2 were measured with the MS2400 TNFR1 and TNFR2 ultrasensitive assay (Meso Scale Discovery, MD, USA). Plasma concentrations of Macrophage Chemo-attractant Protein 1 (MCP1) were measured with the MA2400 Human MCP1 ultrasensitive assay (Meso Scale Discovery, MD, USA). Soluble endothelial selectin (sE-selectin) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described⁹. Plasma Tumor Necrosis Factor α (TNF α) and interleukin 6 (IL6) were measured with an ELISA kit from R&D systems (Abingdon, UK). All samples from one subject were analysed in the same analytical run in duplicate. The intra- and inter-assay variation coefficients were below 10% for all measured parameters.

2.4 *Statistics*

The power to detect a true difference of 0.20 mmol/L in triglyceride concentrations between treatments after adjustment for multiple comparisons was 80%. Normality was checked visually and tested with the Shapiro-Wilk test. Glucose and sE-selectin concentrations were log-transformed to achieve normality. Differences in fasting levels at the end of the intervention periods were compared with a General Linear Model for Univariate ANOVA with treatment as fixed factor and subject number as random factor. Since there were no significant interactions between treatment and gender, and treatment and body weight on the outcome parameters, these parameters were not included in the final model. To adjust for multiple comparisons, a Tukey Honestly Significantly Difference

(HSD) procedure was carried out. A $P < 0.05$ was considered to be statistically significant. Data are presented as mean $\pm$ SD. Statistical analysis was performed using SPSS 15.0 for Windows.

3. Results

3.1 Compliance, dietary intake, bodyweight and blood pressure

The calculated main daily capsule intake was 93% during the fish oil period, 95% during the fenofibrate period and 95% during the placebo period, indicating a good compliance. This was confirmed for the fish oil period, as plasma free EPA and DHA concentrations increased by 358% ($P < 0.001$) and 105% ($P < 0.001$) compared to the placebo period, and by 463% ($P < 0.001$) and 157% ($P < 0.001$) compared to the fenofibrate period, respectively. Total energy intake and the proportions of energy from fat, carbohydrates and protein, and the amounts of fiber, alcohol and cholesterol in the diet did not differ between the treatment groups (data not shown). Furthermore, bodyweight and blood pressure did not change between the treatment periods (data not shown).

Table 2. Effects of fish oil and fenofibrate on serum lipid concentrations and plasma glucose concentrations¹.

	Placebo			Fenofibrate			Fish oil		
Total cholesterol (mmol/L)	6.01	$\pm$	1.32	5.42	$\pm$	1.20*	6.29	$\pm$	1.42#
LDL cholesterol (mmol/L)	3.74	$\pm$	1.14	3.30	$\pm$	1.09*	4.08	$\pm$	1.24**
HDL cholesterol (mmol/L)	1.44	$\pm$	0.41	1.58	$\pm$	0.42*	1.54	$\pm$	0.48
FFA (μ M/L)	396	$\pm$	236	368	$\pm$	237	357	$\pm$	209
Glucose (mmol/L) ²	5.3	$\pm$	0.7	5.4	$\pm$	0.7	5.6	$\pm$	0.7

¹ Values are expressed as mean $\pm$ SD. * $P < 0.05$ versus placebo period, # $P < 0.05$ versus fenofibrate period

² Log transformed concentrations were used for statistical analysis

3.2 Effects on serum lipids and lipoprotein and plasma glucose

Compared to placebo, fenofibrate reduced serum total cholesterol and LDL cholesterol by respectively 9% (-0.59 mmol/L, $P = 0.001$) and 11% (-0.45 mmol/L, $P = 0.004$; **table 2**). Fish oil tended to increase the concentration of total cholesterol ($P = 0.099$) and increased that of LDL cholesterol by 10% (0.34 mmol/L, $P = 0.035$) compared to placebo. Both fenofibrate and fish oil effectively reduced serum triglyceride concentrations by respectively 27% (-0.61 mmol/L, $P < 0.001$) and 13% (-0.34 mmol/L, $P = 0.048$) compared with placebo (**figure 1**).

HDL cholesterol concentrations increased by 11% (0.13 mmol/L, $P=0.013$) after fenofibrate treatment, whereas fish oil tended to increase HDL cholesterol ($P=0.076$) compared to placebo. Compared with fenofibrate treatment, HDL cholesterol ($P=0.737$) and triglyceride concentrations ($P=0.133$) were comparable after fish oil intake, but total cholesterol (0.91 mmol/L, $P<0.001$) and LDL cholesterol (0.78 mmol/L, $P<0.001$) were increased. Concentrations of free fatty acids were not affected by either treatment.

Fish oil tended to raise fasting plasma glucose concentrations compared to placebo (0.24 mmol/L, $P=0.056$) and fenofibrate treatment had no effect ($P=0.721$) compared to placebo. At the end of the intervention period, glucose concentrations between fish oil and fenofibrate treatment ($P=0.250$) did not differ.

Compared to placebo, fenofibrate significantly reduced total VLDL particle numbers (-23 nmol/L, $P=0.001$), in particular large (-2.4 nmol/L, $P=0.003$) and medium VLDL particles (-14 nmol/L, $P=0.001$). Fish oil reduced the number of large VLDL particles (-3.0 nmol/L, $P<0.001$), although the total number of VLDL particles was not affected. It increased however the total number of LDL particles (224 nmol/L, $P=0.005$), but decreased the number of IDL particles (-28 nmol/L, $P=0.016$). For HDL, fenofibrate decreased HDL size (-0.11 nm, $P=0.025$) and increased the number of medium HDL particles (3.1 μ mol/L, $P=0.011$). Fish oil had no overall effect on HDL size, but increased the number large HDL particles (1.5 μ mol/L). Fish oil treatment resulted in higher particle numbers of total VLDL (16 nmol/L, $P=0.02$), medium VLDL (13 nmol/L, $P=0.002$), total LDL (334 nmol/L, $P<0.001$), large LDL (132 nmol/L, $P=0.006$) and small LDL (215 nmol/L, $P=0.043$) compared to fenofibrate treatment (**table 3**). The number of large HDL particles and HDL size were larger (1.8 nmol/L, $P=0.004$ and 0.14 nm, $P=0.004$, respectively). The number of medium HDL particles was smaller after fish oil treatment compared to fenofibrate treatment (-4.8 nmol/L, $P<0.001$).

Table 3. Effects of fenofibrate and fish oil consumption on plasma NMR lipoprofile¹.

	Placebo			Fenofibrate			Fish oil		
<i>Particle numbers</i>									
Total VLDL	63	±	28	40	±	24*	56	±	40 [#]
Large VLDL	4.9	±	3.7	2.4	±	2.6*	1.8	±	3.2*
Medium VLDL	26	±	17	12	±	9*	26	±	24 [#]
Small VLDL	32	±	18	26	±	15	28	±	18
Total LDL	1484	±	554	1374	±	548	1708	±	679**
IDL	55	±	62	40	±	47	27	±	43*
Large LDL	523	±	252	439	±	176	571	±	292 [#]
Small LDL	906	±	614	895	±	497	1110	±	727 [#]
Medium small LDL	191	±	139	190	±	118	223	±	145
Very small LDL	714	±	496	705	±	382	887	±	586 [#]
Total HDL	36	±	8	38	±	6	36	±	8
Large HDL	7.1	±	4.4	6.7	±	3.9	8.6	±	5.3**
Medium HDL	4.5	±	5.4	7.6	±	6.0*	2.8	±	3.4 [#]
Small HDL	25	±	6	24	±	7	25	±	6
<i>Particle sizes</i>									
VLDL	56	±	16	53	±	10	48	±	8*
LDL	21	±	1	21	±	1	21	±	1
HDL	8.9	±	0.5	8.7	±	0.4*	8.9	±	0.5 [#]

¹ Values are means ± SD (n=20). Particle numbers are expressed in nmol/L, except for HDL particles, which are expressed in µmol/L. Particle sizes are expressed in nm.

* P<0.05 versus placebo period,

P<0.05 versus fenofibrate period

3.3 Effects on plasma makers of inflammation and vascular activity

Concentrations of TNFR1, TNFR2 hsCRP, TNF α , IL6, sICAM-1 and sVCAM-1 did not differ between the treatments (table 4). Compared with placebo, the chemokine MCP1, however, increased upon fenofibrate treatment (28 ng/mL, $P=0.034$), but remained unaffected after fish oil treatment ($P=0.204$) (table 4). Further, fenofibrate significantly lowered sE-selectin concentrations compared to both placebo (-4.1 ng/mL, $P=0.034$) and fish oil (-5.7 ng/mL, $P=0.014$), whereas fish oil treatment had no effect compared to placebo ($P=0.932$).

Figure 1. The effects of fish oil and fenofibrate treatment on serum triglyceride concentrations. Data are presented as mean $\pm$ SD. An asterisk indicates a significant effect compared to placebo ($P<0.05$)

Table 4. Effects of fenofibrate and fish oil treatment on markers of inflammation and vascular function¹.

	Placebo			Fenofibrate			Fish oil		
<i>Immune receptors</i>									
TNFR1 (ng/mL)	3.4	±	0.8	3.5	±	0.7	3.4	±	0.6
TNFR2 (ng/mL)	4.3	±	1.1	4.6	±	0.9	4.1	±	1.0
<i>Acute phase protein</i>									
CRP (mg/L)	4.0	±	2.8	3.6	±	1.9	4.3	±	3.7
<i>Cytokines</i>									
TNFα (pg/mL)	2.0	±	0.8	2.0	±	0.6	2.0	±	0.7
IL6 (pg/mL)	2.8	±	1.1	2.7	±	1.1	2.7	±	1.0
MCP1 (ng/mL)	257	±	69	285	±	87*	267	±	64
<i>Adhesion molecules</i>									
ICAM (ng/mL)	275	±	50	304	±	105	267	±	48
VCAM (ng/mL)	440	±	102	451	±	95	425	±	86
E-selectin (ng/mL) ²	76	±	32	72	±	32*	77	±	34 [#]

¹ Values are mean $\pm$ SD. * $P<0.05$ versus placebo period, # $P<0.05$ versus fenofibrate period

² Log transformed concentrations were used for statistical analysis

3.4 Side effects

Fish oil and micronized fenofibrate were well tolerated by all subjects. Fish oil had no significant effects on markers of liver and kidney function (**table 5**) compared to placebo. In contrast, fenofibrate slightly increased ALAT (22 IU/L, $P=0.043$). For 5 out of the 20 subjects values were above the normal range (Laboratory of Clinical Chemistry, University Hospital Maastricht, Maastricht, the Netherlands). Fenofibrate also increased ASAT (13 IU/L, $P=0.016$) and decreased ALP concentrations compared to placebo (-8 IU/L, $P=0.019$). Creatinine concentrations were higher after fenofibrate treatment compared to placebo (9.8 $\mu\text{mol/L}$, $P<0.001$) and fish oil treatment (9.4 $\mu\text{mol/L}$, $P<0.001$). Although γ -GT did not change significantly ($P=0.979$), it slightly exceeded the normal range upon fenofibrate treatment compared to placebo for 4 out of 20 subjects.

Table 5. Effects of fenofibrate and fish oil treatment on markers of kidney and liver function¹.

	Placebo			Fenofibrate			Fish oil			Reference values ²		
Total bilirubin ($\mu\text{mol/L}$)	14	$\pm$	7	12	$\pm$	3	14	$\pm$	5	0	-	17
ALAT (IU/L)	28	$\pm$	8	50	$\pm$	52*	38	$\pm$	27	5	-	45
ASAT (IU/L)	17	$\pm$	7	30	$\pm$	26*	22	$\pm$	10	5	-	35
ALP (IU/L)	75	$\pm$	24	67	$\pm$	23*	73	$\pm$	21	30	-	140
γ -GT (IU/L)	34	$\pm$	10	58	$\pm$	61	35	$\pm$	10	2	-	50
Ureum (mmol/L)	5.0	$\pm$	0.9	5.4	$\pm$	1.3	4.8	$\pm$	0.9	3	-	7
Creatinine ($\mu\text{mol/L}$)	78	$\pm$	12	88	$\pm$	16*	78	$\pm$	13#	53	-	110

¹ Values are mean $\pm$ SD. * $P<0.05$ versus placebo period, # $P<0.05$ versus fenofibrate period

² Reference values from the department of Clinical Chemistry, University Hospital Maastricht, Maastricht, the Netherlands

4. Discussion

Overweight and obese subjects are often characterized by a disturbed lipid and lipoprotein profile, low-grade systemic inflammation, and endothelial dysfunction. A way to improve these metabolic aberrations is by targeting PPAR α . We hypothesized that a dietary intervention with n-3 LCPUFAs, as non-selective PPAR α agonists, could be an alternative for a strong medicinal agonist. Therefore, we directly compared the effects of these n-3 LCPUFAs with those of fenofibrate on a broad range of biomarkers for cardiovascular disease. However, we found that fenofibrate (200 mg/d) and n-3 LCPUFA (3.7 g/d) treatment for

6 weeks did not improve markers for low-grade systemic inflammation and that fenofibrate had more profound effects on plasma lipids and vascular activity compared to fish oil in overweight and obese individuals.

Studies on fenofibrate have shown inconsistent results regarding effects on low-grade inflammation and vascular activity¹⁰⁻¹². We found that fenofibrate reduced sE-selectin concentration compared to placebo and fish oil treatment in overweight and obese subjects. This finding corresponds to that of Hogue *et al.*, who found in type 2 diabetic patients, that micronized fenofibrate (200 mg/d) for 6 weeks reduced plasma sE-selectin, but did not affect concentrations of hsCRP, sICAM1 and sVCAM1¹¹. In contrast, Ryan *et al.* showed in an obese population, that fenofibrate reduced sVCAM1, sICAM1, TNF α , IL6, IL1 β , but did not affect sE-selectin concentrations¹². The reduced sE-selectin concentration as we observed suggests beneficial effects of fenofibrate on vascular activity, since E-selectin is involved in the adherence of leukocytes in the process of atherosclerosis¹³. However, this seems to contradict the observed increase in MCP1 concentrations after fenofibrate treatment compared to placebo, since this chemokine is responsible for attracting monocytes to the injured endothelium¹³. For fish oil, human intervention studies using doses ranging between 1.1 and 6.6 g/d n-3 LCPUFAs are inconsistent and do not often report beneficial effects on inflammatory markers and cellular adhesion molecules¹⁴⁻¹⁸. In that respect, our results agree with those of other studies in obese men^{17, 18} and healthy middle-aged individuals¹⁹, although our sample size may have been too limited to detect statistically significant changes in IL6 and TNF α .

The main well-established effects of fenofibrate and fish oil on plasma lipids are their hypotriglyceridemic effects^{4, 20}. Indeed, we also found that both treatments similarly lowered serum triglyceride concentrations and the number of large triglyceride-rich VLDL particles. These effects have been ascribed to an increased hepatic lipolysis and decreased lipogenesis^{21, 22}, pathways which are under control of PPAR α ². We demonstrated a small increase in HDL cholesterol concentrations after fenofibrate and fish oil treatment, reflected by increases in medium size and large size HDL particles. The increased delivery of surface remnants from the catabolism of VLDL particles, together with a PPAR α -induced expression of apoA1 and apoA2, the main apolipoproteins of HDL, may contribute to the raise in HDL cholesterol²³. Furthermore, PPAR α may stimulate reverse cholesterol transport via induction of ATP Binding Cassette protein A1 (ABCA1)²⁴. Regarding the effects of fish oil and fenofibrate on triglycerides and HDL cholesterol, it is important to note that the degree of these effects largely depend on baseline plasma lipid levels^{4, 25, 26}. In contrast to fenofibrate, fish oil increased LDL cholesterol concentrations. Others have also reported that high dose supplementation of EPA and DHA can raise LDL cholesterol by 5-10%²⁶. In this respect, some groups of subjects may be more sensitive than other groups and it has been suggested that this variability in LDL cholesterol response is related to the apoE4 variant of apolipoprotein E²⁷. For fenofibrate and fish oil treatments, it has been reported that the LDL particle size changes into a more buoyant type, which may be less atherogenic⁵. In our study,

however, this could not be confirmed. Fish oil increased large, small and very small LDL compared to fenofibrate. These findings seem inconsistent in relation to our observed reduction in triglycerides and increase in large HDL particles. When plasma triglycerides are reduced, the proportion or concentration of small LDL particles is expected to be reduced and that of large HDL increased²⁸. We do not have an explanation for these unexpected results.

Finally, we observed a non-significant increase of fasting plasma glucose after fish oil treatment. This agrees with a meta-analysis by Balk *et al.*²⁶, who reported a very small and non-significant average net increase in fasting plasma glucose after treatment with n-3 LCPUFAs.

In summary, although n-3 LCPUFAs and fenofibrate can both activate PPAR α , this study in overweight and obese subjects showed that both fenofibrate (200 mg/d) and fish oil (7.2 g/d, providing 1.7 g/d EPA and 1.2 g/d DHA) treatment for 6 weeks have different effects on cardiovascular risk markers. Both interventions equally lowered serum triglycerides and raised HDL cholesterol, which agrees with few other human studies that have compared side by side the effects of fibrates with those of n-3 LCPUFAs^{5, 29, 30}. Also, as in several other studies²⁶, fish oil treatment increased LDL cholesterol. Both treatments, however, did not improve markers for low-grade systemic inflammation, while fenofibrate had more profound, but apparently conflicting, effects on markers for vascular activity compared to fish oil. Still, like fenofibrate³⁰, LCPUFAs may lower cardiovascular risk through beneficial effects on other cardiovascular risk factors such as blood pressure, arrhythmias and platelet function^{31, 32}.

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CHAPTER 5

Effects of fenofibrate and fish oil on gene expression in peripheral blood mononuclear cells, muscle, and adipose tissue from overweight and obese individuals

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Abstract

The molecular effects of the non-selective dietary PPAR α agonists n-3 LCPUFAs (fish oil) were compared side-by-side with those of the selective synthetic PPAR α agonist fenofibrate on whole-genome gene expression in peripheral blood mononuclear cells (PBMCs), white adipose tissue (WAT), and muscle in overweight and obese subjects. Eleven overweight and obese subjects participated in a randomized, double-blind, placebo-controlled intervention trial and received in random order 3.7 g/d n-3 fatty acids (providing 1.7 g/d EPA and 1.2 g/d DHA), 200 mg fenofibrate or placebo treatment for 6 weeks separated by a wash-out period of at least 2-weeks. Fenofibrate changed 5 of the analysed 56 PPAR α target genes in PBMC, 4 in muscle, and 2 in WAT, whereas fish oil changed 2 genes in WAT only. Both treatments changed 17 out of 25 similar pathways in PBMCs, related to xenobiotic metabolism, biosynthesis of steroids, lipid metabolism, transcription, proteasome function, Th1 immunity, propanoate metabolism and nucleotide metabolism. The majority (18 out of 25) of downregulated pathways in WAT were similarly changed in PBMCs after fish oil and related to lipid metabolism and amino acid metabolism. Fenofibrate and fish oil hardly affected pathways in muscle. In PBMCs, fenofibrate and fish oil only regulated 9 similar genes in metabolic syndrome-related pathways, which were IL25, TICAM1, PRDX2, PTGS1, AGK, FDPS, LDLR, TNNI3, LMAN1 and PTGS1. Overall, 6-weeks treatment with n-3 LCPUFAs and fenofibrate hardly affected the expression of PPAR α target genes in overweight and obese subjects. Pathway analyses suggested that gene expression profiles in PBMCs can provide information about regulated metabolic processes by fish oil and fenofibrate in WAT, but not muscle. Expression of the individual genes, however, hardly overlapped between the tissues.

1. Introduction

The incidence of diet-related diseases, such as cardiovascular diseases and diabetes mellitus type 2, is increasing. These diseases are often linked to the metabolic syndrome, which refers to a cluster of metabolic disturbances related to an abnormal lipid and carbohydrate metabolism, and a pro-inflammatory state of the body. Potential molecular targets to prevent these metabolic disturbances are nuclear peroxisome proliferator-activated receptors (PPARs), which are one of the central regulators of nutrient-gene interactions¹⁻³.

Activation of PPAR α showed that one of its functions is the regulation of genes involved in several aspects of lipid metabolism, including fatty acid uptake through membranes, fatty acid binding in cells, fatty acid oxidation, and lipoprotein assembly and transport⁴. Further, PPAR α may have a beneficial effect on genes involved in (vascular) inflammation and oxidative stress^{1, 5}. In fact, synthetic PPAR α agonists, such as fenofibrate, are already widely used as hypolipidemic drugs. As PPARs function as lipid sensors⁶, nutrition can play a pivotal role in the prevention and treatment of the metabolic syndrome. In this respect, long-chain polyunsaturated fatty acids (LCPUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) deserve attention as effective, but non-selective PPAR activators⁷. Upon binding with fatty acids or a synthetic ligand (e.g. fenofibrate), PPAR bind as a heterodimer with the retinoid X receptor to a PPAR responsive element (PPRE) in enhancer sites of regulated genes to regulate gene transcription⁴.

Comparing fenofibrate to fish oil side-by-side will show how effects on gene expression exerted by a drug relate to those than can be achieved with a dietary intervention. Furthermore, this may reveal important (PPAR α -regulated) genes that reduce the risk of metabolic disturbances. PPAR α is predominantly expressed in the liver, followed by the muscle, intestine and adipose tissue⁸. Since these tissues contribute to the pathology of the metabolic syndrome⁹ and are relatively inaccessible in humans, information on the relationship between gene expression profiles in the easy accessible peripheral blood mononuclear cells (PBMCs) with those in other tissues is essential to monitor the effectiveness of an intervention and to unravel the underlying mechanisms of the metabolic syndrome in specific target tissues. Therefore, the effects of fish oil and fenofibrate on gene expression were investigated in PBMCs, muscle and white adipose tissue from overweight subjects by whole genome microarray analysis.

2. Methods

2.1 Subjects

Subjects with a BMI of at least 27 kg/m² were recruited via posters in the university and hospital buildings and via advertisements in local newspapers. Subjects came to the university for a screening visit. On this visit, fasting blood was sampled for analyses of serum lipids and lipoproteins. In addition, height and body weight

were determined. Furthermore, subjects had to complete a medical and general questionnaire. Exclusion criteria were BMI below 27 kg/m², impairment of kidney and liver function, serum total cholesterol above 8 mmol/L, serum triglycerides above 4 mmol/L, taking medication that could influence the study outcome or could interfere with fenofibrate treatment, use of fish oil supplements, consumption of plant sterol or stanol-enriched food products, having donated blood within 1 month prior to the start of the study, having a diagnosis of any long-term medical condition (e.g. diabetes, cardiovascular diseases, epilepsy) or experiencing strong symptoms of allergy. After screening, twenty-six subjects met all our inclusion criteria and started this study. Subjects received oral and written information about the nature and risk of the experimental procedures before their written informed consent before the start of the study. The study was approved by the Medical Ethical Committee of Maastricht University.

After inclusion of 26 subjects, 6 subjects dropped out and were not included in the analysis (1 man underwent surgery for aneurism, 1 woman had complained about vapours during the placebo period, 1 man and 1 woman did not regularly attend appointments and were excluded, 1 man had a work-related reason, and 1 man had personal reasons). Ten men and ten women completed the trial. For this microarray experiment, the 11 subjects (six men and five women) were studied, who had a complete set at all time points for tissue and PBMC RNA. The baseline characteristics of these 11 subjects were comparable to the remaining 9 subjects who were not included in this sub-study. Subjects were 52 ± 13 y (mean $\pm$ SD), with a bodyweight of 97 ± 9 kg for men and 92 ± 17 kg for women, a BMI of 31 ± 3 kg/m² for men and 33 ± 2 kg/m² for women, and a waist circumference of 117 ± 9 cm for men and 114 ± 8 cm for women. Serum concentrations of total cholesterol were 6.14 ± 1.45 mmol/L, of LDL cholesterol 3.97 ± 1.28 mmol/L, of HDL cholesterol 1.49 ± 0.48 mmol/L, of triglycerides 1.48 ± 0.38 mmol/L, and of glucose 5.51 ± 0.79 mmol/L. Systolic blood pressure was 138 ± 18 mmHg and diastolic pressure was 94 ± 9 mmHg. On average, subjects exhibited 2.5 ± 1.1 components of the metabolic syndrome and 5 out of 11 subjects could be diagnosed as having the metabolic syndrome.

2.2 Study design

The study had a randomized, double-blind, placebo-controlled, crossover design. Each subject enrolled in random order in a fish oil, a fenofibrate and a placebo period for 6 weeks with a wash-out period of at least 2 weeks between the intervention periods. During the fish oil intervention, subjects had to consume 8 fish oil capsules (Marinol C-38™, Lipid Nutrition, Wormerveer, the Netherlands), providing approximately 3.7 g/d n-3 LCPUFA (1.7 g/d EPA and 1.2 g/d DHA, corresponding to 160-240 g fatty fish/d), together with 2 capsules placebo-matching fenofibrate (200 mg/d cellulose). During the fenofibrate period, subjects consumed 2 capsules providing 200 mg/d micronized fenofibrate (Lipanthyl®, Fournier Laboratories, Dijon, France), together with 8 placebo-matching fish oil capsules (containing 80% High Oleic Sunflower Oil (HOSO)). During the placebo

period, subjects received 8 HOSO capsules together with 2 cellulose capsules. Subjects were instructed to ingest half of the capsules before breakfast and the other half before dinner with a glass of water. Subjects were restricted in their fish consumption to a maximum of one portion a week. During the study, subjects recorded any symptom of illness, visits to physician, medication used, alcohol consumption, and any deviations from the protocol in diaries. Body weight was recorded at weeks 0, 5 and 6 of each intervention period and blood pressure was monitored using a sphygmomanometer (Omron M7, CEMEX Medische Techniek BV, Nieuwegein, the Netherlands). Venous blood samples collected in BD vacutainer® tubes (Becton Dickinson Company, NJ, USA) were collected at week 5 and 6. Fasting serum samples were collected at weeks 5 and 6 to measure serum lipids. In week 6 of each intervention period, a fasting EDTA blood sample was collected for the isolation of PBMCs, and a muscle and adipose tissue biopsy was taken for gene expression analysis. At the end of the three intervention periods, energy and nutrient intakes were estimated for the previous 4 weeks using a food frequency questionnaire (FFQ)¹⁰.

2.3 Measurement of serum lipids

Serum samples from weeks 5 and 6 were analysed separately for concentrations of serum total cholesterol (ABX Diagnostics, Montpellier, France), HDL cholesterol (precipitation method; Roche Diagnostics Corporation, Indianapolis, IN), and triglycerides corrected for free glycerol (Sigma-Aldrich Chemie, Steinheim, Germany). Serum LDL cholesterol concentrations were calculated using the formula of Friedewald *et al.*¹¹. After analysis, values were averaged. The free EPA and DHA contents in plasma as a compliance marker, were determined with LC-MS methodology (TNO, Zeist, the Netherlands) as described by Wopereis *et al.*¹² in heparin plasma of week 6 from each period. NaF plasma samples from weeks 5 and 6 were analysed separately for free fatty acids (FFA) using the Wako Nefa C test kit (Wako Chemicals, Neuss, Germany). After analysis, values were averaged.

2.4 PBMC isolation, muscle and adipose tissue biopsies

After sampling, PBMCs were directly isolated from an EDTA anti-coagulated peripheral fasted blood sample at the end (week 6) of each intervention period, through gradient centrifugation using lymphoprep (Nycomed, Oslo, Norway) according to the instructions from the manufacturer. The PBMCs were immediately lysed and homogenised in 1.5 mL Trizol (Invitrogen Life Technologies, Breda, the Netherlands) for RNA stabilisation, immediately frozen in liquid nitrogen, and stored in sterile polypropylene tubes at -80°C until further processing. During the same visit, a muscle and adipose tissue biopsy were taken by a physician. After local anaesthesia (Lidocaine HCL 1%, B.Braun, Melsungen, Germany), a muscle biopsy from the vastus lateralis was taken by passing a 5-mm diameter side-cutting needle through a 7 mm skin incision according to the technique from Bergstrom *et al.*¹³. Following the muscle biopsy, a subcutaneous abdominal adipose tissue biopsy from the peri-umbilical region was taken. Under

local anaesthesia (Lidocaine HCL 1%, B.Braun, Melsungen, Germany), a biopsy was taken with a needle (Braun Medical Sterican needle 2.1*80mm, B.Braun) connected to a vacuum syringe (Hepafix Luerlock, B.Braun). The adipose tissue biopsy was washed with a sterile physiological saline solution to wash off blood and connective tissue. All tissue specimens were immediately frozen in liquid nitrogen and stored in sterile polypropylene tubes at -80°C until further processing.

2.5 *RNA isolation*

Muscle and adipose tissue biopsies were homogenised in trizol using an ultrathurrax (IKA, Labortechnik, Staufen, Germany). Subsequently, RNA from PBMCs, muscle and adipose tissue was isolated from trizol according to a standardized protocol as described by the manufacturer (Invitrogen Life Technologies). Next, RNA was purified and treated with DNase on RNeasy micro columns (Qiagen Benelux B.V., Venlo, the Netherlands), followed by dissolving the RNA in RNase and DNase free water (Invitrogen Life Technologies, Breda, the Netherlands). RNA yield was quantified using the NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was evaluated using the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA, USA) with RNA 6000 nano chips (Agilent Technologies, South Queensferry, UK). Samples with a RIN score > 8.0 were considered to be acceptable for further processing.

2.6 *Microarray processing*

For 11 subjects, muscle, adipose tissue and PBMC RNA samples at the end of each intervention periods could be obtained and was used for microarray processing (99 microarrays in total). Total RNA (300 ng/sample) was labelled with 1-cycle cDNA labelling kit (MessageAmp™ II-Biotin *Enhanced* Kit, Ambion Inc. Austin TX, USA) and hybridized to human whole genome U133 plus 2.0 arrays (Affymetrix Inc, Santa Clara CA, USA). Sample labelling, hybridization to chips and image scanning were performed according to the manufacturer's Gene Chip Expression Analysis Technical Manual (Affymetrix Inc, Santa Clara CA, USA). Array data have been submitted to the Gene Expression Omnibus, accession number GSE27387.

2.7 *Data analysis and statistics*

Differences in fasting levels of serum lipids at the end of the intervention periods were compared with a General Linear Model for Univariate ANOVA with treatment as fixed factor and subject ID as random factor. Since there were no significant interactions between treatment and gender, and treatment and body weight on the outcome parameters, these parameters were not included in the final model. To adjust for multiple comparisons, a Tukey Honestly Significantly Difference (HSD) procedure was carried out. A $P < 0.05$ was considered to be statistically significant. Data are presented as mean $\pm$ SD. Statistical analysis was performed using SPSS 15.0 for Windows.

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 normalisation was performed using quantile normalisation^{14, 15}. Genes with normalised signals below 20 on at least 6 out of 33 arrays from each tissue were excluded for further analyses. Genes were defined as significantly changed when comparison of the normal signal intensities showed a p-value lower than 0.05 in a two-tailed paired t test with Bayesian correction (Intensity Based Modified t-statistics)^{16, 17}. Gene Set Enrichment Analysis was performed was used to focus on groups of genes that shared a biological function or regulation^{18, 19}. Microarray results were analyzed for the expression of 56 known PPAR target genes present on the array (see **additional file 2**), based on the KEGG PPAR signaling pathway (<http://www.genome.jp/kegg/pathway/hsa/hsa03320.html>) and for the expression of genes belonging to key pathways involved in the metabolic syndrome²⁰.

3. Results

3.1 Compliance, dietary intake, body weight and blood pressure

The calculated main daily capsule intake was 95% during the fish oil period, 97% during the fenofibrate period and 96% during the placebo period, indicating a good compliance. This was further confirmed by analysis of plasma free EPA and DHA concentrations, which increased during the fish oil period by 310% ($P<0.001$) and 104% ($P<0.001$) compared to the placebo period, and by 427% ($P<0.001$) and 146% ($P<0.001$) compared to the fenofibrate period, respectively. Compared to placebo, fenofibrate did not change EPA and DHA concentrations ($P=0.904$ and $P=0.920$, respectively). The proportions of energy from fat, carbohydrates and protein, and the amounts of fiber, alcohol and cholesterol in the diet did not differ between the treatments (data not shown). However, total energy intake was lower in the fish oil period compared to the fenofibrate period (-1.2 ± 1.4 MJ, $P=0.017$). Bodyweight and blood pressure did not change between the treatment periods (data not shown).

Figure 1. Fasting plasma triglyceride concentrations after the placebo, fenofibrate and fish oil period. Values are mean SEM ($n=11$). Significant differences: * $P<0.05$ compared to placebo period, # $P<0.05$ compared to fenofibrate period.

3.2 Effect of fenofibrate and fish oil treatment on plasma lipids

As expected, fenofibrate treatment lowered fasting triglyceride concentrations compared to placebo (-0.78 ± 1.04 mmol/L, $P < 0.008$) (figure 1). For fish oil, this effect nearly reached statistical significance (-0.51 ± 0.70 mmol/L, $P = 0.093$). Furthermore, compared to placebo, fenofibrate significantly decreased fasting total cholesterol (from 5.86 ± 1.57 mmol/L to 5.05 ± 0.97 mmol/L, $P = 0.008$) and LDL cholesterol concentrations (from 3.64 ± 1.33 mmol/L to 2.93 ± 0.90 mmol/L, $P = 0.006$) and increased HDL cholesterol concentrations (from 1.32 ± 0.36 mmol/L to 1.54 ± 0.38 mmol/L, $P = 0.002$). Fish oil increased HDL cholesterol concentrations compared to placebo, but this effect just did not reach statistical significance (from 1.32 ± 0.36 mmol/L to 1.45 ± 0.45 mmol/L, $P = 0.057$). Total cholesterol and LDL cholesterol were non-significantly increased after fish oil treatment (from 5.86 ± 1.57 mmol/L to 5.96 ± 1.63 mmol/L, $P = 0.658$ and from 3.64 ± 1.33 to 3.73 ± 1.34 , $P = 0.473$). FFA concentrations were non-significantly decreased after fenofibrate and fish oil treatment (from 374 ± 222 μ M to 342 ± 262 μ M ($P = 0.500$) and to 316 ± 214 μ M ($P = 0.121$), respectively).

Figure 2. Venn diagrams of number of upregulated and downregulated genes in >8 subjects, which overlap between fenofibrate and fish oil treatment in PBMCs (A), muscle (B) and adipose tissue (C). Genes with a P value < 0.05 were considered to be significantly regulated compared to the placebo period.

3.3 Differential expressed genes in PBMC, muscle and adipose tissue transcriptome

In PBMCs, fish oil treatment significantly changed the expression of 537 genes and fenofibrate treatment that of 1445 genes with 106 overlapping genes. Of these overlapping genes, 84 changed into the same direction (figure 2). Compared with PBMCs, fewer genes were differentially expressed in muscle and adipose tissue. In muscle tissue, 486 genes were differentially regulated after fenofibrate treatment and 287 genes after fish oil treatment, while all 48 overlapping genes changed into the same direction. In contrast to PBMCs and muscle tissue, fish oil had a more pronounced effect on gene expression in adipose tissue. Fish oil consumption resulted in the differential expression of 441 genes compared to 259 genes after fenofibrate treatment. The large majority of overlapping genes (25 out of 29) between both treatments changed into the same direction. Overall, there was relatively little overlap in regulated genes between fish oil and

fenofibrate treatment in any of the tissues. There was also very little overlap in regulated genes between tissues upon fenofibrate and fish oil treatment and the expression of none of the genes were similarly changed in all three tissues for either treatment (**figure 3**).

Figure 3. Venn diagrams of overlap in regulated genes between the tissues after fenofibrate (A) and fish oil (B) treatment. Genes with a P value < 0.05 were considered to be significantly regulated compared to the placebo period.

3.4 Pathway analysis

A summary of regulated processes in PBMCs is given in figures 4 and 5. Fish oil and fenofibrate treatment regulated 25 common pathways in PBMCs, of which 17 were showed shared downregulation and 15 were regulated in opposite directions (data only partly shown in **figure 4 and 5**). Fenofibrate and fish oil overlapped in the downregulation of pathways involved in xenobiotic metabolism, biosynthesis of steroids, cholesterol biosynthesis cell cycle, transcription, oxidative phosphorylation, fatty acid synthesis, proteasome function, Th1 immunity, propanoate metabolism and nucleotide metabolism. Pathways related to integrin-mediated cell adhesion, interleukin signaling, gap junction and roles of beta-arrestin-dependent recruitment of src kinases in GPCR signalling, Interleukin 3 and interleukin 6 signaling were upregulated after fenofibrate treatment and downregulated after fish oil treatment. Fenofibrate (down)regulated only 3 pathways in WAT, which were related to FAS signaling (NES -1.74), intrinsic prothrombin activation (NES -1.68) and natural killer cell mediated cytotoxicity (NES -1.60). Fish oil treatment affected many more pathways in WAT compared to fenofibrate (**figure 6**). Predominantly immune and inflammatory processes were upregulated, while processes related to fatty acid metabolism and oxidative phosphorylation were downregulated. Of the 25 downregulated pathways in WAT, 18 pathways were similarly regulated in PBMCs after fish oil treatment (data partly shown in **figure 6**). Fenofibrate treatment had little effects on regulated pathways in muscle. Only genes related to statin pathway (NES -1.98), P53 signaling (NES -1.93), chondroitin sulphate biosynthesis (NES -1.88), ribosome (NES -1.87) and leukocyte transendothelial migration (NES -1.85) were downregulated. Also fish oil had only very minor effects on pathways in muscle. Upregulated GSEA processes were proteasome (NES 2.11), proteasome degradation (NES 1.89), integrin signaling and interleukin 9 signaling (NES 1.84), and downregulated processes were DNA replication reactome (NES -1.87), O-glycan biosynthesis (-1.85) and antigen processing and presentation (NES -1.76).

Figure 4. Selection of pathways regulated in PMBCs after fenofibrate treatment identified by Gene Set Enrichment Analysis (FDR q-value <0.25, highest Normalized Enrichment score (NES)). The NES reflects the degree to which a gene set is overrepresented at the top (upregulated) or bottom (downregulated) of the ranked gene list and is corrected for gene set size. Light grey bars indicate an overlap of pathways regulated by fenofibrate and fish oil treatment in PMBCs.

Figure 5. Selection of pathways regulated in PMBCs after fish oil treatment identified by GSEA (FDR q-value <0.25, highest Normalized Enrichment score (NES)). The NES reflects the degree to which a gene set is overrepresented at the top (upregulated) or bottom (downregulated) of the ranked gene list and is corrected for gene set size. Light grey bars indicate an overlap of regulated pathways regulated between fenofibrate and fish oil treatment in PMBCs.

3.5 Expression of PPAR target genes and metabolic syndrome-related genes

Fenofibrate significantly changed 5 of the analysed 56 PPAR target genes in PBMC, 4 genes in muscle, and 2 genes in WAT (**table 1** and **additional file 2**). Fish oil did not change any of the known PPAR target genes in PBMC and muscle. In WAT, 2 PPAR target genes were significantly downregulated (**table 1**). PPAR α did not significantly change in any of the tissues due to the treatments. However, PPAR γ was upregulated in muscle after fenofibrate treatment, while PPAR δ was downregulated in WAT after fish oil treatment.

Figure 6. Selection of pathways regulated in WAT after fish oil treatment identified by GSEA (FDR q-value <0.25, highest Normalized Enrichment score (NES)). The NES reflects the degree to which a gene set is overrepresented at the top (upregulated) or bottom (downregulated) of the ranked gene list and is corrected for gene set size. Light grey bars represent processes similarly regulated in PBMCs after fish oil and fenofibrate treatment

Specific genes belonging to key pathways²⁰ involved in the metabolic syndrome were regulated by fenofibrate and fish oil in PBMCs (**tables 2 and 3**), of which fenofibrate affected the expression of a larger number of those genes. Although both treatments regulated specific genes in these pathways, only the change in expression of 9 genes was shared. These genes were IL25, TICAM1, PRDX2, PTGS1, involved in inflammation and/or oxidative stress; AGK, FDPS and LDLR involved in lipid metabolism and transport; and TNNI3, LMAN1 and PTGS1 involved in vascular function and coagulation. Except for PTGS1, all these genes changed into the same direction.

Table 1. Significantly changed genes from a set of 64 known PPAR target genes¹ in PBMC, WAT and muscle upon fenofibrate and fish oil treatment.

Gene name	Entrez ID	Description	FC	P-value
PBMC				
<u><i>Fenofibrate</i></u>				
NR1H2	10062	nuclear receptor subfamily 1, group H, member 3	1.11	0.01
CYP27A1	1593	cytochrome P450, family 27, subfamily A, polypeptide 1	1.09	0.04
ILK	3611	integrin-linked kinase	1.08	0.02
RXRA	6256	retinoid X receptor, alpha	1.07	0.04
ACSL5	51703	acyl-CoA synthetase long-chain family member 5	-1.05	0.03
<u><i>Fish oil</i></u>				
- No change in expression of PPAR target genes -				
WAT				
<u><i>Fenofibrate</i></u>				
APOCIII	345	apolipoprotein C-III	1.23	0.00
SORBS1	10580	sorbin and SH3 domain containing 1	1.07	0.02
<u><i>Fish oil</i></u>				
RXRA	6256	retinoid X receptor, alpha	-1.07	0.04
PPARD	5467	peroxisome proliferator-activated receptor delta	-1.11	0.01
MUSCLE				
<u><i>Fenofibrate</i></u>				
PPARG	5468	peroxisome proliferator-activated receptor gamma	1.14	0.03
PDPK1	5170	3-phosphoinositide dependent protein kinase-1	1.06	0.04
ILK	3611	integrin-linked kinase	-1.07	0.03
PLTP	5360	phospholipid transfer protein	-1.18	0.01
<u><i>Fish oil</i></u>				
- No change in expression of PPAR target genes -				

¹ See additional file 2 for the complete list of PPAR α target genes

Table 2. Significantly changed genes ($P < 0.05$) in PBMCs after fish oil treatment in selected pathways adversely affected in the metabolic syndrome.

Fish oil				
Pathway	Gene name	Entrez ID	Description	FC
Energy homeostasis	GHSR	2693	growth hormone secretagogue receptor	1.10
	CCL1	6346	chemokine (C-C motif) ligand 1	1.14
	TLR9	54106	toll like receptor 9	1.14
	IL19	29949	interleukin 19	1.13
	IL6	3569	interleukin 6	1.11
Inflammation / oxidative stress	TNFSF18	8995	tumour necrosis factor ligand family member 18	1.11
	DEFB1	1672	beta defensin 1	1.11
	BTLA	151888	B and T lymphocyte attenuator	1.10
	IL25 ¹	64806	interleukin 25	1.08
	IL4R	3566	interleukin 4 receptor	1.08
	TICAM1 ¹	148022	toll like receptor adaptor molecule 1	1.08
	IL17RB	55540	interleukin 17 receptor B	1.07
	MASP2	10747	mannan-binding serine peptidase 2	1.07
	PRDX2 ¹	7001	peroxiredoxin 2	-1.08
	PTGS1 ¹	5742	cyclooxygenase-1	-1.11
	CIQA	712	complement Cq1 subcomponent subunit A	-1.21
Lipid metabolism / Transport	ELA3A	10136	chymotrypsin-like elastase family member 3A	1.09
	APOM	55937	apolipoprotein M	1.08
	ADIPOR1	51094	adiponectin receptor 1	1.07
	AGK ¹	55750	acylglycerol kinase	-1.06
	INSIG1 ²	3638	insulin-induced gene 1	-1.08
	FDPS ^{1,2}	2224	farnesyl diphosphate synthetase	-1.08
	LDLR ¹	3949	LDL receptor	-1.13
	FADS1	3992	fatty acid desaturase 1	-1.15
	DHCR24	1718	24-dehydrocholesterol reductase	-1.16
	CAV1	857	caveolin 1	-1.17
Glucose homeostasis / insulin signalling	SCT	6342	Secretin	1.17
	GHSR	2693	growth hormone secretagogue receptor	1.10
	SLC37A4	2542	glucose-6-phosphate translocase	1.06
	ZFP106	64397	zinc finger protein 106	-1.06
Vascular function / coagulation	TNNI3 ¹	7137	troponin I 3	1.14
	F11	2160	coagulation factor XI	1.14
	AGT	183	Angiotensinogen	1.07
	LMAN1 ¹	3998	lectin, mannose-binding, 1	-1.08
	PTGS1 ¹	5742	prostaglandin endoperoxidase synthase 1	-1.11

¹ Overlap between fenofibrate and fish oil treatment, ² Shared regulation with WAT

Table 3. Significantly changed genes in PBMCs after fenofibrate treatment in selected pathways adversely affected in the metabolic syndrome.

Fenofibrate				
Pathway	Gene name	Entrez ID	Description	FC
Energy homeostasis	LEPR	3953	Leptin receptor	1.12
	CXCL5	6374	chemokine (C-X-C motif) ligand 5	1.46
	CXCL9	4283	chemokine (C-X-C motif) ligand 9	1.26
	PTGS1 ¹	5742	prostaglandin endoperoxidase synthase 1	1.24
	GPX3	2878	glutathione peroxidase 3	1.23
	CXCL10	3627	chemokine (C-X-C motif) ligand 10	1.21
	SOD2	6648	superoxide dismutase 2	1.14
	FPR1	2357	formyl peptide receptor 1	1.13
	NFAM1	150372	NFAT activating protein with ITAM motif 1	1.13
	SBN2	22904	strawberry notch homologue	1.12
Inflammation / oxidative stress	TGFB1	7040	transforming growth factor beta 1	1.11
	BDKRB1	623	bradykinin receptor B1	1.11
	TICAM1 ¹	148022	toll like adapter molecule	1.10
	TICAM2	353376	toll like adapter molecule 2	1.08
	IL25 ¹	64806	interleukin 25	1.07
	MSRA	4482	methionine sulfoxide reductase A	1.07
	PRDX6	2071	peroxiredoxin 6	-1.05
	GCLC	2729	glutamate-cysteine ligase, catalytic subunit	-1.06
	MIF	4282	macrophage migration inhibitory factor	-1.08
	PRDX2 ¹	9588	peroxiredoxin 2	-1.10
	INFG	3458	interferon gamma	-1.25
	ABCA1	19	ATP binding cassette transporter A1	1.24
	LIPC	3990	hepatic lipase	1.24
	DGAT2L3	158833	diacylglycerol-O-acetyltransferase-2 like 3	1.16
	MGLL	11343	monoacylglycerol lipase	1.15
Lipid metabolism / transport	LPLR1	4035	low density lipoprotein receptor related protein 1	1.13
	APOE	348	apolipoprotein E	1.12
	OSBP2	23762	oxysterol binding protein 2	1.11
	CRAT	1384	carnitine-O-acetyltransferase	1.09
	PLB1	151056	phospholipase B1	1.08
	APOB48R	55911	apolipoprotein B48 receptor	1.07
	SLC27A3	11000	long chain fatty acid transport protein	1.07
	FDFT1	2222	farnesyl diphosphate farnesyltransferase 1	-1.06
	ACSM3	6296	acyl-CoA synthetase long-chain family member 3	-1.07
	SC5DL	6309	sterol C5 desaturase	-1.07
	CROT	54677	carnitine octanoyltransferase	-1.08
	ACAT2	39	acetyl-Coenzyme A acetyltransferase	-1.09
	FDPS ¹	2224	farnesyl diphosphate synthase	-1.09
	ACADSB	36	acyl-Coenzyme A dehydrogenase, short/branched chain	-1.11
	LDLR ¹	3949	low density lipoprotein receptor	-1.11
	AGK ¹	55750	acylglycerol kinase	-1.13

Fenofibrate				
Pathway	Gene name	Entrez ID	Description	FC
Glucose homeostasis / insulin signalling	GK ²	2710	glucose kinase	1.22
	GAA	2548	glucosidase, alpha; acid	1.11
	SLC37A2	219855	glycerol-3-phosphate transporter	1.11
	GRB10	2887	growth-factor receptor bound protein 10	1.07
	MGAT4A	11320	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N acetylglucos-aminyltransferase, isoenzyme A ¹	-1.06
Vascular function / coagulation	PTGS1 ¹	5742	prostaglandin endoperoxidase synthase 1	1.24
	GPIBA	2811	glycoprotein Ib (platelet), alpha polypeptide	1.24
	GP9	2815	glycoprotein IX (platelet)	1.21
	F13A1 ²	2162	coagulation factor XIII, A1 polypeptide	1.21
	TBXAS1	6916	thromboxane A synthase 1 (platelet)	1.13
	TNNI3 ¹	7137	troponin I type 3	1.14
	P2RX1	5023	purinergic receptor P2X, ligand-gated ion channel, 1	1.14
	SERPINE1	5054	plasminogen activator inhibitor type I	1.11
	F12	2161	coagulation factor XII (Hageman factor)	1.11
	F2RL1	2150	coagulation factor II (thrombin) receptor-like 1	1.10
	RENBP	5973	renin binding protein	1.09
	LMAN1 ¹	3998	lectin, mannose-binding, 1	-1.08

¹ Overlap between fenofibrate and fish oil treatment, ² Shared regulation with muscle

For WAT, fish oil also regulated genes in key pathways belonging to the metabolic syndrome and shared the upregulation of APOC3 and APOM with fenofibrate treatment (**table 4**). Furthermore, fenofibrate upregulated the VLDLR gene in WAT. In muscle, fish oil downregulated genes involved in inflammation, such as IL1R1, IL13, TICAM2 and CXCL14, and affected genes involved in glucose metabolism and insulin signaling, such as IRS2, PIK3R1. Compared to fish oil, fenofibrate treatment also downregulated CXCL14 in muscle. Fenofibrate downregulated genes involved in inflammation, such as MIF and C3. Genes in other pathways related to the metabolic syndrome were not affected.

Table 4. Significantly changed genes in WAT after fish oil treatment in selected pathways adversely affected in the metabolic syndrome.

Fish oil				
Pathway	Gene name	Entrez ID	Description	FC
Energy homeostasis	PPARD	5467	peroxisome proliferator activated receptor delta	-1.11
	NRI1	8204	nuclear receptor interacting protein 1	-1.14
Inflammation / oxidative stress	CXCL10	3627	chemokine (C-X-C motif) ligand 10	1.53
	CD180	4064	CD180 molecule	1.53
	TNFSF4	7292	tumour necrosis factor ligand family member 4	1.42
	FCER1G	2207	Fc fragment of IgE, high affinity 1, receptor for; gamma polypeptide	1.25
	SAMHD1	25939	SAM domain and HD domain 1	1.17
	CD4	920	CD4 molecule	1.09
	TIRAP	114609	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	1.05
	CXCL12		chemokine (C-X-C motif) ligand 12	-1.10
	C3	718	complement component 3	-1.11
	APOC3 ¹	345	apolipoprotein CIII	1.11
Lipid metabolism / Transport	ACAD8	27034	acyl-Coenzyme A dehydrogenase family, member 8	-1.08
	HMGCS1		3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-1.10
	PPARD	5467	peroxisome proliferator activated receptor delta	-1.11
	ACADSB	36	acyl-Coenzyme A dehydrogenase, short/branched chain	-1.11
	APOD ²	347	apolipoprotein D	-1.12
	FDPS ³	2224	farnesyl diphosphate synthase	-1.13
	NSDHL	50814	NAD(P) dependent steroid dehydrogenase-like	-1.14
	DCI	1632	dodecenoyl-Coenzyme A delta isomerase	-1.16
	SCARB1	949	scavenger receptor class B, member 1	-1.16
	ACSS2	55902	acyl-CoA synthetase short-chain family member 2	-1.24
	INSIG1	3638	insulin-induced gene 1	-1.25
	PNPLA3	80339	patatin-like phospholipase domain containing 3	-1.31
Glucose homeostasis / insulin signalling	AACS	65985	acetoacetyl-CoA synthetase	-1.38
	PDK3	5165	pyruvate dehydrogenase kinase, isozyme	1.14
	INSR	3643	Insulin receptor	-1.07
	PPARD	5467	peroxisome proliferator activated receptor delta	-1.11
	PGM5	5239	phosphoglucomutase 5	-1.17
	APOH	350	apolipoprotein H	1.14
	F10	2159	coagulation factor X	-1.16
Vascular function / coagulation	MMP2	4313	matrix metalloproteinase 2	-1.16

¹ Overlap between fenofibrate and fish oil treatment, ² Shared regulation with muscle, ³ Shared regulation with PBMCs

4. Discussion

Six weeks of treatment with fenofibrate exerted only very minor effects on PPAR target genes in PBMCs, white adipose tissue or muscle. Serum triglyceride concentrations, however, were decreased. This may indicate that fenofibrate exerts most of its effects in the liver. Fenofibrate can be considered as a pro-drug, which is rapidly absorbed in the gastrointestinal tract²¹. During absorption,

fenofibrate undergoes rapid hydrolysis and is converted into its pharmacologically active form, fenofibric acid. After that, fenofibric acid is quickly distributed and cleared by the intestine, kidney and liver²¹. This may explain the lack of direct effects on PPAR α target gene expression in PBMCs, muscle and adipose tissue. Fish oil supplementation increases the EPA and DHA content of plasma and tissues and may therefore influence gene expression in larger variety of tissues²². However, there was also a lack of effect on the expression of PPAR α target genes after fish oil treatment. In a 26-weeks study conducted in elderly subjects, fish oil treatment (1.8 g EPA+DHA per day) even decreased PPAR α target genes²³. A postprandial study with a 33 g n-3 LCPUFA-containing high fat shake in healthy subjects increased only a small number of PPAR α target genes in PBMCs²⁴. In contrast to nutritional interventions, 24h fasting resulted in pronounced increases in PPAR α target gene expression in PBMCs, possibly due to tremendous increases in plasma endogenous free fatty acids²⁵. Thus, the effect of n-3 LCPUFAs on PPAR α gene expression in PBMC seems to be largely influenced by plasma concentrations of FFA. Based on these previous studies, the changes in gene expression we observed upon fenofibrate and fish oil treatments are probably indirect and the result of longer term adaptation of the body and systemic changes. Furthermore, other transcription factors than PPAR α that are also involved in various metabolic processes can be influenced by both treatments, such as LXR by fenofibrate²⁶ and PPAR γ , PPAR δ , LXR, FXR, SREBPs, HNF4 α and NF κ B by n-3 LCPUFA²⁷. Indeed, we observed that fenofibrate treatment upregulated LXR- α expression and its target gene ABCA1 in PBMCs. Fish oil treatment did not change the expression of transcription factors in PBMCs, WAT and muscle.

Clearly, muscle tissue and WAT are no primary target tissues for fenofibrate, since in these tissues almost no biological, as defined by GSEA, were changed after fenofibrate treatment. The predominant target organ for fenofibrate is the liver, where PPAR α is also more abundantly present as compared to muscle and WAT⁸. Fish oil exerted only very minor effects in muscle and exerted its strongest effects in WAT. The majority (18 out of 25) of downregulated pathways in WAT were comparable to the pathways downregulated in PBMCs after fish oil. These pathways were mainly related to lipid metabolism (synthesis and oxidation of fatty acids, biosynthesis of cholesterol and triglyceride synthesis) and amino acid metabolism (degradation branched chain aminoacids (BCAA)). The decrease in lipogenic pathways may suggest that n-3 LCPUFAs shift the balance in adipose tissue from fat storage towards mobilization of fatty acids for oxidation in muscle and liver, as reported by other studies²⁸⁻³⁰. However, we did not find any changes in the regulation of pathways involved in lipid mobilization. Also, pathways involved in lipid oxidation in muscle were not changed. The precise mechanisms of lipid metabolism in human adipose tissue is very complex and remains to be further elucidated. The apolipoprotein C3 (APOC3) gene, a natural and strong inhibitor of adipose tissue lipoprotein lipase (LPL) activity, was upregulated upon fish oil and fenofibrate treatment in WAT. LPL enhances fatty acid uptake from triglyceride in

adipose tissue. In mice studies, presence of APOC3 has been proposed to be an interesting target to reduce excessive triglyceride storage, diet-induced obesity, and insulin resistance^{31, 32}. On the contrary, polymorphism studies revealed that deficiency of the APOC3 gene in humans was associated with reduced triglyceride levels and reduced arterial plaque formation³¹. Therefore, it is uncertain if the effect of fish oil and fenofibrate on APOC3 expression in WAT can be regarded as beneficial. Surprisingly, fish oil regulated pathways involved in amino acid metabolism in WAT. This is a less recognized role of adipose tissue compared to its role in lipid metabolism. Fish oil decreased the expression of genes involved in the degradation of BCAA. This suggest a reduced degradation of BCAA in the fasting state to preserve BCAA for glucose production and ketogenesis and prevent their irreversible conversion into lipids for storage^{33, 34}.

Fish oil and fenofibrate treatment regulated 32 common pathways in PBMCs, of which 17 downregulated after both treatments and 15 were regulated in a different direction. However, there was almost no overlap between the treatments in regulated genes. This indicates that fenofibrate and fish oil treatment affect different target genes, which may result in commonly regulated metabolic pathways. Pathways with a shared downregulation were mainly involved in lipid metabolism, cell cycle, tissue turn-over and amino acid metabolism. Shared pathways, which were upregulated by fenofibrate treatment and downregulated by fish oil treatment, were mainly involved in cell adhesion, tissue morphogenesis and inflammation. Surprisingly, fenofibrate treatment upregulated pathways and several genes involved in eicosanoid metabolism and coagulation. This seems to contrast the reported anti-thrombotic and anti-platelet activities of fenofibrate³⁵.

Since PPAR α is involved in the regulation of multiple metabolic pathways, fish oil and fenofibrate treatment may be a way to alleviate metabolic disturbances often seen in subjects with the metabolic syndrome. Our results show that only 9 similar genes relevant for the metabolic syndrome were regulated by fenofibrate and fish oil in PBMCs. Among those overlapping genes, the LDL receptor (LDLR) was downregulated by both treatments. Studies with lipid lowering interventions have indeed shown that PBMCs change the expression of the LDLR gene^{36, 37}. The decreased LDLR expression we observed upon fish oil treatment is in line with results from the study from Bouwens *et al.*²³. Reduced LDLR expression could be a reaction to the increased serum and probably also increased intracellular cholesterol concentration upon fish oil treatment³⁸. In contrast, the decreased LDLR expression upon fenofibrate was unexpected, since plasma cholesterol concentrations were significantly reduced. The other overlapping genes between fish oil and fenofibrate treatment were interleukin 25 (IL25), toll-like receptor adaptor molecule 1 (TICAM1), PTGS1 and peroxiredoxin-2 (PRDX2) involved in inflammation and oxidative stress; acyl glycerol kinase (AGK) and farnesyl diphosphate synthase (FDPS) involved in lipid metabolism and transport; and troponin I type 3 (TNNI3), lectin-mannose binding 1 (LMAN1) and PTGS1 involved in vascular function and coagulation.

Gene expression profiling can be an interesting tool to examine the metabolic

state of a target tissue or to study changes reflected by external stimuli, such as nutrition. In humans, the inaccessibility of most target tissues complicates such a study. Therefore, PBMCs may be of interest to serve as surrogate cells as they are more readily accessible. In this respect, it has been shown that these cells are able to reflect changes caused by diet³⁹, medicine⁴⁰, toxic exposure⁴¹ and exercise⁴². Our study showed that PBMCs are indeed able to respond to interventions as shown by specific gene expression profiles of fish oil and fenofibrate treatment compared to placebo. Although some common pathways were regulated, there was however only very little overlap in gene expression changes between PBMCs and muscle or WAT, which clearly indicates that gene expression is tissue and organ specific. Only a very limited number of studies reported single gene expression changes simultaneously in PBMCs and another (target) tissue. In humans, gene expression in PBMCs have been compared to liver, focussing on LDL receptor⁴³, HMG-CoA expression^{43, 44}, ABC transporters⁴⁵ correlated between liver and PBMCs. It seems that in PBMCs, expression levels of these genes involved in lipid metabolism correlate with those in liver. PBMCs might be good surrogate cells for these genes. In our study, the genes encoding for insulin-induced gene 1 (INSIG1) and FDPS were both downregulated in PBMCs and WAT after fish oil treatment. It has been suggested that INSIG1 contributes to the triglyceride lowering effect of PPAR α activation by inhibition of sterol-regulatory binding element 1c (SREBP1c) activation⁴⁶. The downregulation of this gene may reflect a feedback mechanism to the decreased triglyceride concentrations by fish oil and fenofibrate treatment. FDPS, a SREBP-responsive gene, has been associated with decreased cholesterol and TG levels in animal studies^{47, 48}. In addition, the lipolytic GK gene was upregulated in both PBMCs and muscle after fenofibrate treatment, a key enzyme in regulating glycerol uptake and metabolism.

Overall, 6-weeks treatment with n-3 LCPUFAs and fenofibrate hardly affected PPAR α target genes in overweight and obese subjects. Pathway analysis, however, suggested that gene expression profiles in PBMCs can provide information about regulated metabolic processes by fish oil and fenofibrate in WAT, but not muscle. Expression of the individual genes, however, hardly overlapped between the tissues.

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CHAPTER 6

Differential effects of fenofibrate and fatty acids on NF κ B activity in HepG2 cells

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Abstract

Peroxisome proliferator-activated receptor α (PPAR α) is known as a lipid sensor and can inhibit the pro-inflammatory transcription factor nuclear factor B (NF κ B). Therefore, targeting PPAR α with fatty acids is of interest to reduce hepatic manifestations of the metabolic syndrome in which low grade-inflammation plays an important role. For this reason, we evaluated the effects of different fatty acids (palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6), α -linolenic acid (C18:3, n-3), eicosapentaenoic acid (EPA; C20:5, n-3) and docosahexaenoic acid (DHA; C22:6, n-3)) and fenofibric acid, a synthetic PPAR α agonist, on the interrelationships between PPAR α and NF κ B activities a human liver cell model in vitro. We showed that overexpression of PPAR α in human liver cells reduced basal NF κ B expression in a dose-dependent manner. Fenofibric acid, C18 fatty acids stearic acid, oleic acid, linoleic acid and α -linolenic acid, and EPA could increase PPAR α activity. In contrast, palmitic acid hardly affected and DHA decreased PPAR α activity at 50 and 100 μ M. Fenofibric acid inhibited NF κ B activity, and this effect requires the presence of PPAR α . However, almost all fatty acids inhibited NF κ B activity independent of PPAR α . Exceptions were oleic acid at 1 and 10 μ M and α -linolenic acid at 1 μ M, showing NF κ B activation in the absence of PPAR α and NF κ B inhibition in the presence of PPAR α . In conclusion, activation of PPAR α by fenofibrate in liver cells can inhibit NF κ B activity. In addition, the modulation of NF κ B activity by fatty acids is fatty acid specific, where PPAR α counteracted the activation of NF κ B by oleic and α -linolenic acid. In contrast, the NF κ B inhibitory effect of palmitic acid, stearic acid, linoleic acid, EPA and DHA in liver cells did not depend on PPAR α .

1. Introduction

Low-grade systemic inflammation is often present in overweight and obese subjects and contributes to an increased risk for developing the metabolic syndrome and associated diseases¹. A potential molecular target to counteract this low-grade pro-inflammatory state is peroxisome proliferator activated receptor α (PPAR α). PPAR α is a member of the nuclear receptor super family of ligand-activated receptors and well-known for its role in the expression of genes involved in lipid metabolism². However, PPAR α may also modulate inflammatory responses by physically interacting with the pro-inflammatory transcription factor nuclear factor κ B (NF κ B), thereby forming inactive complexes³. NF κ B activation is a crucial step in the inflammatory response and regulates the expression of pro-inflammatory cytokines, chemokines and adhesion molecules. Furthermore, PPAR α can indirectly control inflammatory responses by increasing the expression of I κ B α and I κ B kinase (IKK), which are known to inhibit NF κ B signaling^{4,5}. I κ B α is a protein that binds to NF κ B and prevents its translocation to the nucleus, whereas IKK prevents degradation of I κ B α .

PPAR α can be activated by synthetic ligands, such as drugs of the fibrate class. However, dietary fatty acids can also activate PPAR α , which is illustrated by the fact that PPARs are frequently described as lipid sensors^{6,7}. It has been suggested that especially polyunsaturated fatty acids (PUFAs) with 18-20 carbon atoms and 3-5 double bonds such as α -linolenic acid (α -LA) and eicosapentaenoic acid (EPA), as well as monounsaturated fatty acids (MUFAs) bind to and activate PPARs^{7,8}. Docosahexaenoic acid (DHA) was found to be a less potent PPAR α activator. The effects of different fatty acids on NF κ B activation and the role of PPAR α herein have not been studied before. Therefore, the aim of the present study was to explore *in vitro* in human liver cells the effects of fenofibric acid and different fatty acids on the interrelationships between PPAR α and NF κ B activities. Oleic acid, linoleic acid, α -linoleic acid were used to examine varying degrees of unsaturation, palmitic acid for its described pro-inflammatory effect, and the fish fatty acids EPA and DHA for their suggested anti-inflammatory properties.

2. Material and methods

2.1 Reagents

Bovine serum albumine (BSA; endotoxin and fatty acid free), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6), α -linolenic acid (C18:3, n-3), eicosapentaenoic acid (EPA; C20:5, n-3) and docosahexaenoic acid (DHA; C22:6, n-3) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fenofibric acid (FeAc) was kindly provided by Fournier Laboratories (Dijon, France). Minimal Essential Medium (MEM), trypsin, penicillin streptomycin (PS), sodium pyruvate (SP) and non-essential amino acids (NEAA) were obtained

from Invitrogen Corporation (Paisley, UK), and Fetal Calf Serum (FCS; South-American) from Greiner Bio-one (Frickenhausen, Germany).

2.2 *HepG2 cell culture, transfection, fatty acid experiments and luciferase assay*

HEPG2 cells were kindly provided by Mabtech (Nacka Strand, Sweden). Cells were cultured in MEM containing glucose and L-glutamine, which was further supplemented with 10% heat-inactivated FCS (v/v), 1% PS (v/v), 1% SP (v/v), and 1% NEAA (v/v). Cells were cultured at 37 °C in a 5% CO₂/95% O₂ (v/v) humidified atmosphere and separated twice a week, when they had reached 80% confluency. For passage, cells were first washed with phosphate buffered saline (PBS), followed by addition of trypsin-0.03% ethylenediaminetetraacetic acid (EDTA) (v/v). For each experiment, HepG2 cells were seeded in 24-well plates with a density of 0.2×10^6 cells per well in a total volume of 1 mL MEM. Twenty-four hours after seeding, at $\pm 40\%$ confluency, cells were transiently transfected with the appropriate plasmids using Fugene 6 Transfection Reagent according to the user manual (Roche Diagnostics, Basel, Switzerland). To study the effects of PPAR α agonists on NF κ B transactivation, cells were transfected with a 4kB-TK-luciferase (NF κ B luciferase reporter) plasmid, which was kindly provided by Dr. David Gius (Radiation National Oncology Section, National Cancer Institute, Bethesda, MD, USA). To measure PPAR transcriptional activity, cells were transfected with a luciferase reporter pGL3 vector containing PPAR response elements (PPREs) as described by Gilde *et al.*⁹ In addition, all cells were co-transfected with a human PPAR α (hPPAR α) expression construct. After 5 hours, FCS-free MEM containing the fatty acids was added to the cells, which were subsequently incubated for 24 hours. Fatty acids were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the medium did not exceed 0.33% (v/v). Fatty acids (1, 10, 50 and 100 μ M) were added to MEM with 0.1% BSA and pre-incubated at 37°C for 45 minutes to enable coupling of fatty acids to BSA. FeAc (300 μ M) was added and incubated for 24 hours as a positive control for PPAR α transactivation. A condition with only BSA served as a negative control. Each compound was tested in quadruplicate. After 24 hours, cells were lysed on ice with 100 μ L reporter lysis buffer per well (Promega, Madison, WI, USA) and stored at -80°C. Luciferase activity was measured according to the manufacturer's instruction (Promega) and expressed relative to protein content (Bio-rad assay, Bio-rad, Hercules, CA, USA).

2.3 *Statistics*

Values for PPAR α and NF κ B reporter activity were analyzed by one-way ANOVA. If significant, the effects for each fatty acid were compared at each concentration to the negative control using the post hoc Bonferroni test. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). *P*-values < 0.05 were regarded to be statistical significant.

3. Results

3.1 Effects of PPARα on NFκB transactivation

A PPRE-luciferase reporter construct was used to determine the activity of PPARα. HepG2 cells transfected with only the PPRE-luciferase reporter showed almost no PPRE-mediated luciferase activity, even not after incubation with the strong PPARα agonist FeAc (**figure 1**). Co-transfection of HepG2 cells with the PPARα expression construct resulted in a 4-fold increase in PPRE-mediated luciferase activity compared to basal PPRE activity. PPARα activation could be enhanced 12-fold by the addition of the PPARα agonist FeAc (**figure 1**).

Figure 2 shows that increasing the transfected amount of the PPARα construct dose-dependently decreased NFκB luciferase activity (**Figure 2**). This decrease became statistically significant at PPARα concentrations of 0.06 μg and more, and NFκB luciferase activity was almost completely abolished at a concentration of 0.1 μg PPARα.

Figure 1. Effect of FeAc (300 μM) in HepG2 cells transfected with the PPRE luciferase reporter with and without co-transfection with the PPARα expression construct. The control without PPARα and without FeAc was set to 1. Values significantly different from control (p<0.05) are indicated with an asterisk (*).

Figure 2. Dose response curve of hPPARα (0.02, 0.04, 0.06, 0.08 and 0.10 μg) using the PPRE luciferase reporter construct. The control condition without PPARα was set to 1. Values significantly different from control (P<0.05) are indicated with an asterisk (*).

Figure 3. The effect of fatty acids C16:0, C18:0, C18:1, C18:2, C18:3, C20:5 and C22:6 in a concentration ranging from 1 - 100 μ M, on PPAR α transactivation. The transactivation properties of fatty acids are shown relative to the transactivation of PPAR α alone. The condition with BSA (negative control) is set at zero % activation. Values significantly different from $P < 0.05$ are indicated with an asterisk (*).

3.2 Effects of fatty acids on PPAR α transactivation

None of the tested concentrations of palmitic acid (C16:0) was able to transactivate PPAR α (**figure 3**). In contrast, stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and α -linolenic acid (C18:3) activated PPAR α at 50 μ M and oleic acid also at 100 μ M. The long-chain fish fatty acids EPA (C20:5) and DHA (C22:6) hardly influenced PPAR α activity, except for a significantly reduced PPAR α activity at 100 μ M of DHA.

Figure 4. Effect of FeAc (300 μ M) on NF κ B transactivation in presence and absence of PPAR α . The control condition with PPAR α and without FeAc was set to 1. Values significantly different from control ($p < 0.05$) are indicated with an asterisk (*).

3.3 *Effects of FeAc and fatty acids on NF κ B transactivation in absence and presence of a PPAR α*

In the absence of the PPAR α construct, FeAc did not influence NF κ B transactivation. Interestingly, after co-transfection of the PPAR α construct, FeAc significantly inhibited NF κ B transactivation by 82% (**figure 4**).

To test the effects of different fatty acids, HepG2 were transfected with the NF κ B luciferase reporter, first in the absence of the PPAR α expression construct. Palmitic acid significantly inhibited NF κ B activity at concentrations of 10, 50 and 100 μ M, whereas stearic acid had no effect (**figure 5a**). The activity of the NF κ B reporter also increased at 1 and 10 μ M of oleic acid, whereas NF κ B activation was shown for linoleic acid at 50 and 100 μ M, and for α -linolenic acid at 1 μ M. For the fish fatty acids, EPA significantly inhibited NF κ B activity at 50 and 100 μ M and DHA at 100 μ M. After co-transfection with PPAR α , the NF κ B stimulating effect of oleic acid at 1 and 10 μ M was completely abolished (**figure 5b**), as was also seen for 1 μ M α -linolenic acid. Furthermore, stearic acid significantly inhibited NF κ B activity at 50 and 100 μ M. For the other fatty acids in combination with PPAR α , there were only marginal differences in the NF κ B modulating effects when compared to the situation without PPAR α co-transfection. Correlations between the effects of the fatty acids on PPAR α activity with effects on NF κ B activity did not reach statistical significance

Figure 5. The effect of fatty acids C16:0, C18:0, C18:1, C18:2, C18:3, C20:5 and C22:6 in a concentration ranging from 1 - 100 μ M, on NF κ B transactivation in the absence (A) and presence (B) of PPAR α . The condition with BSA (negative control) was set at zero % activation. Values significantly different from the negative control ($P<0.05$) are indicated with an asterisk (*).

4. Discussion

Low-grade inflammation is often present in subjects with the metabolic syndrome. This pro-inflammatory state can contribute to hepatic manifestations related to this syndrome, such as non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH)^{1, 10, 11}. There is evidence from in vitro experiments in aortic smooth muscle cells that PPAR α can dampen the inflammatory response by preventing NF κ B translocation to the nucleus^{4, 5}. We have extended these findings by showing for the first time cells that in HepG2 cells activation of PPAR α by FeAc can also inhibit NF κ B activity. This is interesting, because an increased NF κ B activity is a characteristic of patients with NASH^{12, 13}. In contrast to fenofibrate, the effects of PPAR α in HepG2 cells on NF κ B by fatty acids was, if anything at all, marginal.

It is thought that PPAR α can be activated by natural ligands, such as fatty acids. So far, activation of PPAR α by fatty acids has almost exclusively been studied with ligand binding assays or by using only a GAL4-fused ligand binding domain of PPAR α ^{7, 8}. However, to detect true fatty acid ligands and activators of PPAR α , it may be preferred to use the fully functional transcription factor. Using this approach, we here show that fatty acids with 18 carbon atoms are activators of PPAR α in HepG2 cells. In contrast, palmitic acid and the fish oil fatty acids EPA and DHA were poor PPAR α activators. These results are largely in agreement with the findings of Mochizuki *et al.*, who examined the effects of fatty acids on PPAR α binding and activity in a GAL-4 fused PPAR α luciferase system in a colon cell line (CaCo2)⁸. They found that especially linoleic acid, α -linolenic acid, γ -linolenic acid, arachidonic acid and EPA stimulated PPAR α . Less activity was seen after incubation with palmitic acid and oleic acid. In addition, DHA hardly affected PPAR α transcriptional activity in that study, although there was an increased binding of DHA to PPAR α ⁸.

In contrast to fenofibrate, PPAR α seems to play only a limited role in NF κ B inhibition by fatty acids in HepG2 cells. The fatty acids palmitic, stearic and linoleic acids, EPA and DHA inhibited NF κ B activity in the absence of PPAR α . Remarkably, palmitic acid dose-dependently reduced NF κ B activity, while oleic and α -linolenic acids were the only fatty acids capable of activating NF κ B. Joshi-Barve *et al.* reported an increased expression and production of neutrophil chemoattractant interleukin-8 in combination with an increased DNA binding activity of the pro-inflammatory transcription factors NF κ B and AP-1 after stimulation of HepG2 cells with 300 μ M palmitic acid¹⁴. It was not reported if this increased DNA binding was also followed by an increased transactivation of NF κ B by palmitic acid.

From in vitro studies in muscle cell lines, it is known that the saturated fatty acid palmitic acid stimulates NF κ B activity and consequently the production of the pro-inflammatory cytokines IL6 and TNF α ¹⁵⁻¹⁷. Thus, the effects we observed may be liver-specific. It has been hypothesized by Pasparakis *et al.* that NF κ B can have distinct roles in different tissues, illustrated by contrasting responses of tissues to NF κ B inhibition¹⁸. The inhibition (or activation) of NF κ B in skin, intestinal epithelial cells and liver parenchymal cells forms the trigger for an immune response, and

NF κ B-competent immune cells will further drive the inflammatory response¹⁸. Contrasting, inhibition of NF κ B in central nervous system cells, pancreas and skeletal muscle cells did not inflict an inflammatory response¹⁸. The reason for potential tissue-specific effects of fatty acids on activation of NF κ B could relate to differences in expression of toll-like receptor (TRL) types^{19,20}. TRLs can be activated by fatty acids and play an important role in the regulation of NF κ B activity. TRLs can be differentially modulated by the different fatty acids in various tissues^{21, 22}. How this can explain our observed NF κ B activating capacity of oleic acid and low dose α -linoleic acid in liver cells warrants further investigation. For macrophages and human embryonic kidney cells, it has been reported that unsaturated fatty acids (including oleic acid) inhibited TRL and subsequent NF κ B activation²³.

DHA and EPA are n-3 LCPUFAs from fish oil and are known to reduce inflammation and to exert cardioprotective effects²⁴. However, the mechanisms behind these effects are not clear. It is suggested that these effects arise from inhibition of NF κ B²⁵. We have shown that EPA and DHA may reduce NF κ B activation, at least in liver cells. These effects were independent of PPAR α . Also for the other fatty acids, with the exception of oleic acid and low dose α -linoleic acid, the presence of PPAR α had only minor influences on NF κ B activity. It could be that the NF κ B-suppressing effects of these fatty acids are PPAR α -independent. Since fatty acids are non-specific PPAR ligands, modulation of NF κ B expression could possibly also occur via PPAR γ ^{26,27} instead of PPAR α . However, it is not likely that anti-inflammatory effects of PPAR γ agonists can simply be attributed to PPAR activation²⁸. Research with the specific PPAR γ agonist rosiglitazone failed to demonstrate NF κ B-related inflammatory gene expression in peripheral blood mononuclear cells of diabetes patients²⁹ or showed only an effect in a study with a dose beyond the pharmacological level⁴. Fatty acids, especially EPA and DHA, may also influence inflammation via the earlier mentioned TRLs, modulation of liver X receptor (LXR) α or β , hepatic nuclear factor α (HNF4 α) or nod-like receptors (NRLs)^{28, 30}. Hypothetically, it could also be that fatty acids act more directly on the NF κ B signaling cascade. These direct effects may involve reduced I κ B phosphorylation via decreased mitogen activated protein kinases (MAPKs) or reduced IKK activity^{25, 31-33}. Alternatively, PPAR α -mediated inhibition of NF κ B via PPAR α is masked in our experiments by counteracting factors or mechanisms. More in vitro experiments and in vivo research with tissue-specific knock-out models are therefore necessary to elucidate the molecular mechanisms behind the NF κ B-mediated anti-inflammatory effects of fatty acids.

In conclusion, the presence and activation of PPAR α by fenofibrate in liver cells can inhibit the activation NF κ B. In addition, the modulation of NF κ B activity by fatty acids is fatty acid specific, where PPAR α counteracted the activation of NF κ B by oleic and α -linolenic acid. In contrast, the NF κ B inhibitory effect of palmitic, stearic, linoleic acid, EPA and DHA in liver cells seem independent of PPAR α and involve other biological relevant pathways or potential counteracting mechanisms.

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CHAPTER 7

General discussion

The incidence of the metabolic syndrome is rapidly increasing world-wide among adults and also among adolescents and children^{1,2}. This syndrome is characterized by a cluster of metabolic abnormalities, such as obesity, disturbed lipid and glucose metabolism and hypertension, which predisposes to the development of type 2 diabetes (T2D) and cardiovascular diseases (CVD)³. Therefore, there is an urge to prevent or reverse the metabolic abnormalities associated with the metabolic syndrome and to prevent the development of these chronic diseases.

Nutrition can play a pivotal role in disease prevention. Dietary interventions mainly focus on the very early phase of disease development, when there is a pre-disease state with metabolic stress, but no clinical manifestations of the disease, as in case of the metabolic syndrome (**figure 1**). In this way, early symptoms can be reversed to normalize metabolic homeostasis. Nutrition and pharmacological approaches to prevent or ameliorate metabolic stress and the metabolic syndrome can be complementary. However, drugs are more often applied at a later stage when clinical symptoms already occur.

Figure 1. Development of the metabolic syndrome (adapted from Van der Greef *et al.*⁴). A nutritional approach can be applied to prevent the metabolic syndrome and normalize metabolic homeostasis. A pharmacological approach is more often applied after the onset of disease in order to treat the disease. Dietary intervention studies using a nutrigenomics approach may reveal early biomarkers of disease in order to define better dietary strategies for disease prevention. Interesting targets for prevention of the metabolic syndrome are PPARs, which can be activated by pharmacological as well as dietary interventions.

Unfortunately, the underlying molecular mechanisms of the metabolic syndrome are largely unknown, which makes it difficult to develop effective prevention strategies. Dietary intervention studies using a nutrigenomics approach to study the effects of a diet or dietary compound at the gene, protein and metabolite level may reveal early biomarkers of effect. This will be helpful in determining and applying effective intervention strategies to prevent the

metabolic syndrome by reversing metabolic stress back into normal homeostasis. The search for these molecular biomarkers is complicated due to the relative inaccessibility of target tissues. Therefore, it is also important to elucidate if the readily accessible peripheral blood mononuclear cells (PBMCs) can be used as a surrogate.

There is an interesting and considerable overlap in molecular targets between nutritional and pharmacological intervention strategies. In this light, peroxisome proliferator activated receptors (PPARs), such as PPAR α and PPAR γ , deserve attention. These transcription factors can be activated by pharmacological ligands, such as fibrates and thiazolidinediones, respectively, but also by dietary fatty acids. Most often, pharmacological approaches are more powerful and dietary interventions lead to more subtle molecular changes. Comparing a pharmacological and a nutritional approach to target PPARs may therefore reveal how gene expression effects exerted by a drug relate to those achieved by a dietary intervention. In this way, dietary intervention strategies can be explored for prevention of the metabolic syndrome or as an alternative to drugs to improve metabolic abnormalities associated with the metabolic syndrome.

The major objective of the studies described in this thesis was to examine the role of PPARs in various metabolic processes and to identify a potential role for nutrition in modulating these transcription factors. A second objective was to evaluate the suitability of PBMCs as surrogate cells for target tissues to reflect PPAR-related gene expression and gene expression in general. For this, we studied the effects of both synthetic PPAR agonists and natural PPAR agonists on a wide range of metabolic risk markers. As synthetic ligands we used rosiglitazone (PPAR γ) and a micronized form of fenofibrate (PPAR α), which are commonly used, potent agonists. We used n-3 fatty acids from fish oil as natural non-selective dietary ligands of PPARs. Except for the effects on serum lipids and lipoproteins, we were particularly interested in the effects on inflammation markers and markers of vascular activity. In addition, we investigated if effects on these parameters were reflected by changes in gene expression. This chapter will provide a comprehensive overview of the main results of the conducted studies, which will be discussed and put into a broader perspective.

1. Targeting PPARs to modulate lipid and glucose metabolism

The first PPAR, PPAR α ⁵, was discovered in 1990, followed by the discovery of the existence of 3 isotypes, PPAR α , PPAR γ and PPAR δ ⁶. Since then, numerous studies have been conducted and PPARs received widespread attention as therapeutic targets in T2D and CVD. PPAR α , γ and δ are key regulators of lipid and glucose metabolism. The research in this thesis focuses on PPAR α and PPAR γ . Synthetic ligands of PPAR α and PPAR γ are already used in the clinical practice. Fibrates have been used for a long time to treat hyperlipidemia, due to their potent triglyceride (TG) lowering action via PPAR α . Thiazolidinediones, synthetic ligands

for PPAR γ , have been used to treat T2D patients by improving their insulin sensitivity. Interestingly, it was also discovered that PPARs can be activated by natural dietary ligands⁷. Fatty acids and their metabolites can act as metabolic regulators by controlling the activity of these lipid-sensing transcription factors. Therefore, the research described in this thesis was conducted to examine the role of PPAR α and PPAR γ in various metabolic processes and to identify a potential role for nutrition in modulating these transcription factors.

1.1. *Effects of PPAR agonists on circulating metabolic parameters*

The majority of the effects we found on lipid and glucose metabolism for the studied PPAR ligands (**figure 2**) were consistent with the existing literature. In our study, 8 weeks rosiglitazone treatment (8 mg/d) improved insulin sensitivity in T2D making these patients again as insulin sensitive as healthy reference subjects (**chapter 3**). The mechanism underlying this effect is related to an increased glucose uptake and a stimulation of adipocyte differentiation, which creates an increased number of smaller and more insulin sensitive adipocytes. This latter way leads to a preferential shuttling of free fatty acids (FFA) to subcutaneous tissue, which reduces the lipid load in muscle and liver, making these tissues more insulin sensitive. This was also shown by an increased insulin-mediated reduction in FFA and TG. A consequence of the promotion of adipocyte differentiation by rosiglitazone can be an expansion of subcutaneous adipose tissue and subsequent weight gain⁸. Weight gain, however, was not apparent in our study after 8 weeks of rosiglitazone treatment in T2D patients. Rosiglitazone treatment improved serum lipids, by increasing high-density lipoprotein (HDL) cholesterol and by reducing concentrations of FFA and TG. Most likely, the increase in HDL cholesterol is a result of non-selective or indirect effects of rosiglitazone. It is possible that this agonist can also weakly activate PPAR α , increase the expression of transcription factor Hepatic Nuclear Factor 4 α (HNF4 α), or decrease inflammation followed by an elevated ApoA2 production responsible for the increase in HDL cholesterol⁹.

Figure 2. Summary of the effects on serum lipids and lipoprotein particles, plasma glucose and insulin of A) PPAR γ agonist rosiglitazone in type 2 diabetic (T2D) subjects, B) PPAR α agonist fenofibrate in overweight and obese subjects and C) n-3 fatty acids from fish oil as natural, non-specific PPAR ligands in overweight and obese subjects. FFA: free fatty acids, HDL: high-density lipoprotein, IDL: intermediate-density lipoprotein, LDL: low-density lipoprotein, TCH: total cholesterol, TG: triglycerides, VLDL: very low-density lipoproteins.

Consistent with clinical studies with pre-diabetic and diabetic subjects, we did not detect an effect of the synthetic PPAR α agonist fenofibrate on plasma glucose and insulin levels in overweight and obese subjects. This clearly illustrates the isotype-specific functions of PPARs. PPAR α predominantly regulates lipid metabolism and has potent hypotriglyceridemic effects. Indeed, our study showed that fenofibrate treatment (200 mg/d) for 6 weeks effectively improved the serum lipid profile in overweight and obese subjects (**chapter 4** and **figure 2**). The mechanisms underlying these effects are related to an increased clearance of lipids from the circulation. This increased clearance is due to lowering of hepatic ApoCIII production and stimulation of lipoprotein-lipase mediated lipolysis via PPAR α ¹⁰. The combination of increased uptake and catabolism of fatty acids and TG, and reduced TG synthesis will lower serum TG and large TG-rich very low density lipoprotein (VLDL) particles^{11, 12}. There are multiple proposed mechanisms for the increase in HDL upon fenofibrate, including an increased production of apolipoproteins A1 (ApoA1) and A2, and the induction of cholesterol transporter ABCA1^{9, 13, 14}. Normally when the number of large VLDL particles is reduced, the

proportion of small atherogenic low density lipoprotein (LDL) is expected to be reduced and that of large HDL increased¹⁵. However, we could not demonstrate this in our study and showed even a slight decrease in HDL size upon fenofibrate. We do not have an explanation for this discrepancy in findings, but the substantial decrease in number of LDL particles will in this case most likely outweigh the lack of beneficial effect on LDL particle size. Overall, fenofibrate effectively improved the serum lipid profile in overweight and obese subjects.

The main well established effect of fish oil is its TG lowering effect. Many studies have reported that this action of fish oil is mediated via the same PPAR α dependent mechanism as described for fenofibrate¹⁶⁻¹⁸. On the contrary, there are also studies showing TG lowering in fish oil fed PPAR α -deficient mice and mice on PPAR α antagonists and fish-oil diet fed mice, suggesting the presence of a PPAR α -independent pathway in lipid metabolism^{19,20}. Regardless of the exact mechanism, our study also confirmed that fish-oil treatment (1.2 g/d docosahexaenoic acid (DHA) and 1.7 g/d eicosapentaenoic acid (EPA)) for 6 weeks resulted in a marked 13% decrease in TG in obese and overweight subjects compared to the placebo group on high oleic sunflower oil (HOSO) (**chapter 4**). However, this TG-lowering effect was accompanied by a significant increase in LDL cholesterol of 10%. Although the effects of fish oil on LDL cholesterol are inconsistent between the many studies, meta-analyses have shown that LDL cholesterol can indeed increase by 5-10% in normolipidemic and hyperlipidemic subjects at high dosages of EPA and DHA^{21, 22}. The impact of fish oil on lipoprotein subclasses has been less extensively studied, but a shift in size distribution from smaller dense to more buoyant and less atherogenic LDL particles has been reported^{23, 24}. However, we did not find a change in LDL particle size distribution upon fish oil treatment. The reported effects of fish oil on glucose metabolism are inconsistent. We saw a tendency towards increased fasting plasma glucose levels. This may be due to stimulation of hepatic glucose production or to decreasing hepatic insulin secretion by fish oil²⁵. Overall, fish oil has beneficial effects on TG levels. However, since fish oil increased LDL cholesterol without improving the quality of LDL particles, caution should be taken using extreme dosages of fish oil in subgroups susceptible for developing CVD.

1.2 *Metabolic genes and pathways affected by PPAR agonists*

Microarray analyses were performed on PBMCs, muscle and white adipose tissue (WAT) samples from eleven subjects who received 6-week treatments with placebo, fish oil and fenofibrate treatment (**chapter 5**). These tissues are relevant in the multi-factorial etiology of the metabolic syndrome and studying gene expression in these tissues could reveal important (PPAR α -regulated) genes and pathways implicated in metabolic risk reduction by pharmacological and dietary treatment. Muscle and white adipose tissue are clearly no primary target tissues of PPAR α , given the tissue distribution of PPAR α ²⁶ and the fact fenofibrate hardly affected any pathways in these tissues. In PBMCs, fenofibrate and fish oil downregulated the pathway involved in fatty acid synthesis, in

line with the (non-significant) reduction in plasma FFA and reduction in TG. Furthermore, fenofibrate downregulated cholesterol biosynthesis, which is in correspondence with the observed reduction in plasma LDL cholesterol. Yet, some lipid metabolic pathways changed in another direction than expected, such as the downregulation of pathways involved in lipid oxidation.

Although fasting serum TGs were reduced by fenofibrate and fish oil treatment, this did not correspond to a change in expression of the PPAR α target gene lipoprotein lipase, which codes for the enzyme involved in the TG-lowering effects of fibrates²⁷. Furthermore, other PPAR α target genes hardly changed in PBMCs, muscle, and WAT. It was at least expected that the well known lipid-related PPAR α target genes, such as fatty acid transporter protein (FATP), AcylCoA synthase and carnitine palmitoyltransferase (CPT) I and II would have been upregulated by fenofibrate and fish oil treatment^{10, 28-30}. To speculate, these PPAR α target genes may still be involved to explain the TG lowering effect observed after fish oil and fenofibrate treatment, although not in the tissues and cells measured. In this case, these effects are probably almost exclusively mediated by modulating PPAR α target genes in the liver. However, the relevance of the intestine should not be underestimated considering the emerging role of this organ in cholesterol metabolism³¹⁻³³. It may also be possible that changes in gene expression we observed in PBMCs are less related to a direct effect of fenofibrate on these cells, but are secondary to changes in plasma metabolites originating from the liver. For fish oil, human long-term and postprandial studies with fish oil also showed no or hardly any changes in PPAR α target genes^{34, 35}. In contrast, high levels of FFA resulting from 24-hour fasting did change PPAR α target genes³⁶. There is a possibility that TG-lowering by fish oil treatment is regulated by PPAR α -independent mechanisms as PPAR α antagonists were not able to influence the lipid-lowering by fish oil in diet-induced obese mice²⁰.

In addition to lipid metabolic pathways, fenofibrate and fish oil downregulated certain mitochondrial metabolic pathways in PBMC and WAT, including oxidative phosphorylation, branched chain amino acid (BCAA) degradation and Krebs cycle (**figure 3**). The decrease in mitochondrial bioenergetics after fenofibrate treatment seems in line with that in rodent studies³⁷⁻⁴⁰. This effect is probably caused by a direct interference of fenofibrate with mitochondrial respiration³⁹. This may result in the inhibition of oxidative phosphorylation and production of energy in the form of adenosine triphosphate (ATP). Subsequent accumulation of fatty acids and the induction of fatty acylCoA oxidase activity may provide a means to trigger the proliferation of peroxisomes. Substantial increased peroxisome-proliferation in the liver has been implicated in hepatocarcinoma in rodents. Probably, this effect is independent of PPAR α . Studies in liver biopsies from humans on fibrate therapy for 2-156 months showed an inconclusive increase in peroxisome proliferation (<1.5 fold), which indicates that humans are not susceptible to PPAR α -mediated cancer of liver⁴¹. On the other hand, the effects of fish oil and fenofibrate on mitochondrial metabolic pathways have been rarely studied in human cell lines or humans. It is not valid to directly translate gene expression data to physiological

outcomes, especially when measured in PBMCs as surrogate cells, but it may be worthwhile to further investigate the effects of fish oil and fenofibrate on mitochondrial metabolic pathways in humans or human cell lines.

Figure 3. Inter-linked mitochondrial metabolic pathways are downregulated by fish oil and fenofibrate in PBMCs and by fish oil in WAT in overweight and obese subjects. BCAA: branched chain amino acids, FF: fenofibrate, FO: fish oil, LCFA: long-chain fatty acids, SCFA: short chain fatty acids, TCA: tricarboxylic acid.

2. Inflammation and metabolic diseases: role for PPARs?

2.1 PPAR agonists and inflammation control

Atherosclerosis is a process in which lipid and fibrous material is deposited in the arterial wall in the setting of a low-grade inflammatory state⁴². The metabolic syndrome is also characterized by a chronic state of low-grade inflammation and associated with an increased CVD risk^{43, 44}. PPARs have been shown to play a role in critical CVD-related processes, but the impact of and the mechanism behind PPAR agonists treatment and CVD outcomes remains under debate⁴⁵. It has been reported that PPAR agonists are able to inhibit pro-inflammatory transcription factors, such as NFκB, leading to a downregulation of pro-inflammatory genes and reduction of circulating markers of inflammation⁴⁶. Indeed, we showed in a mechanistic in vitro study in human liver cells that fenofibrate and different fatty acids are able to inhibit NFκB activity, although the effect of fatty acids seems to be independent of PPARα (**chapter 6**). Despite the potency of PPAR agonists to inhibit NFκB activity, we did not observe potent anti-inflammatory effects of PPAR agonists that can be linked with CVD reduction in our human intervention studies (**figure 4**). Only a very few inflammatory markers were inconsistently affected by rosiglitazone (**chapter 3**) and fenofibrate (**chapters 4 and 5**) treatment, showing both pro- as anti-inflammatory effects. If there is a role for synthetic PPAR agonists in CVD risk reduction, this is probably mainly due to their insulin-sensitizing and/or hypolipidemic effects. The n-3 fatty acids EPA

and DHA have been reported to reduce TG levels at higher dosages (EPA+DHA 3 g/d), similar as given in our study. For cardiovascular risk reduction, however, a lower dosage (EPA+DHA: 1 g/d) that hardly affects serum TGs is advised. This recommendation was based on an observed 20-30% reduction of CVD mortality in long-term secondary prevention trials. Despite the high dosage of EPA and DHA that we used and the large hypotriglyceridemic effect that was shown, we did not demonstrate in overweight and obese subjects any improvements in any measure of circulating marker of inflammation after 6-weeks fish oil supplementation (**chapter 4**). These results are in correspondence with findings from other studies with dosages ranging from 1.1 and 6.6 g/d of n-3 LCPUFAs⁴⁷⁻⁵¹. In line, a recent study by Skulas-Ray *et al.* did not show any effects of a 8-weeks treatment low (0.85 g/d) and high (3.5 g/d) dosage of EPA+DHA on inflammation, endothelial function in healthy persons with moderate hypertriglyceridemia in a placebo-controlled, randomized double-blind cross-over trial, despite the 27% TG-lowering by the high dosage⁵². Furthermore, they found no changes in expression of inflammatory cytokine genes in PBMCs of these patients. Except for a downregulation of the TNF-NF κ B pathway, we could also not show any changes in inflammatory pathways or individual inflammatory genes in PBMCs after a 6-weeks treatment with 2.9 g/d of EPA+DHA treatment in overweight and obese subjects. Evidence that n-3 LCPUFAs from fish oil reduce the risk of CVD is strong, but it is becoming more and more clear that this is not mediated by an improvement of systemic markers of inflammation and vascular activity. Instead, other mechanisms, such as anti-arrhythmic and anti-coagulant properties, are more likely to underlie the CVD protective effect of n-3 LCPUFAs from fish oil. Furthermore, the effect of fish oil also depends on the dose⁵³⁻⁵⁵.

Figure 4. Summary of effects on fasting markers of inflammation and vascular activity of rosiglitazone in T2D patients and of fenofibrate and fish oil in overweight and obese subjects.

Illustrated is the link between the measured markers in relation to the atherosclerotic process. CRP: C-Reactive protein, E-selectin: endothelial selectin, ICAM: intracellular adhesion molecule 1, IL6: interleukin 6, LDL: low-density lipoprotein, MCP1: macrophage chemoattractant protein 1, VCAM: vascular cellular adhesion molecule.

2.2 Hyperinsulinemia and inflammation

The question about the role of elevated insulin levels becomes relevant in light of the low-grade pro-inflammatory state associated with insulin resistance, the metabolic syndrome and T2D. Both hyperinsulinemia⁵⁶⁻⁵⁸ and hyperglycemia⁵⁹⁻⁶¹ have been held responsible for promoting inflammation. Elevated blood glucose levels have been shown to trigger inflammation markers, including IL6, TNFα, and CRP⁶². This response is more pronounced in diabetic patients compared to non-diabetic controls. Furthermore, the increase of these markers seems to be independent of insulin production. It has even been shown that insulin has the potency to exert anti-inflammatory effects via inhibition of NFκB⁶³. Indeed, our study also showed that a supra-physiological concentration of insulin during the hyperinsulinemic clamp is not per se related to an increase in inflammatory markers, and even to lowered MCP1 levels in T2D patients and healthy controls (**chapter 3**). Therefore, it is likely that hyperglycemia and not hyperinsulinemia is responsible for the pro-inflammatory state seen in insulin resistant and T2D subjects.

2.3 *Inflammation and PPARs: a gut feeling?*

There is no doubt that obesity is associated with a subclinical inflammatory state and predisposes to T2D and CVD. However, the factors responsible for the initiation and maintenance of chronic inflammation are not yet clear. More and more evidence points towards an important role of the intestinal environment in metabolic diseases. Besides an altered lipid and glucose metabolism, obesity and T2D are also characterized by changes in gut permeability and changes in intestinal microbiota. Disruption of the gut barrier may lead to bacterial translocation and enhanced uptake of endotoxins (lipopolysaccharides (LPS)) originating from gram-negative bacteria from the lumen. Furthermore, obesity is characterized by a shift in the quantity and proportion of two dominant gut bacteria: *Bacteroides* and *Firmicutes*. In the course of obesity, the number of *Firmicutes* increases over the number of *Bacteroides*⁶⁴. Although the most favorable gut microflora composition and causality has not yet been defined, changes in microbiota may be important, because it can determine endogenous LPS absorption from gram-negative bacteria in the gut. Endotoxins follow the route of dietary fat, as they are absorbed along with lipids⁶⁵. This is a way in which a chronic high-fat diet can contribute to the development of endotoxemia, leading to a low-grade systemic inflammatory state⁶⁶. LPS can trigger certain receptors, such as NF κ B and TLR 4, inducing a signaling cascade leading to the release of pro-inflammatory cytokines. Thereby, low-grade inflammation is maintained and can even be worsened. This metabolic endotoxemia can increase the risk of diabetes and atherosclerosis. Indeed, it has been shown that T2D patients have higher LPS levels than controls⁶⁷.

Striving for a beneficial gut microbiota composition and optimal gut integrity could provide alternative and interesting ways for metabolic risk management in light of the metabolic syndrome. However, more (long-term) clinical trials will be needed to unravel the complex relationship between the intestinal environment and the development of obesity, T2D and CVD. Although evidence is limited, PPARs could also play a potential role herein. It has been shown that rodents subjected to restrained stress show an increase in intestinal permeability, a decrease the absorption capacity, and an increase the level of inflammatory markers^{68, 69}. There are indications from rodent studies that PPAR α and PPAR γ can control intestinal barrier function⁷⁰, and prevent intestinal inflammation and dysfunction^{68, 69}.

3. Nutritional versus pharmacological approach to target PPARs

3.1 *Can a nutritional approach compete with pharmacological approach?*

Fish oil (2.9 g/d EPA+DHA) and fenofibrate (200 mg/d) exerted similar hypotriglyceridemic effects after 6-weeks supplementation in overweight and obese subjects (**chapter 4**). It is likely that this effect is mediated via a comparable mechanism of PPAR α activation^{71, 72}, but PPAR α -independent effects cannot be excluded¹⁹. In contrast to fish oil, fenofibrate exerted also significant beneficial

effects on LDL- and HDL-cholesterol concentrations. However, it should be noted that fenofibrate is also able to raise LDL cholesterol levels in a situation of severe hypertriglyceridemia⁷³. Fenofibrate and fish oil both lacked consistent beneficial effects on markers of inflammation, questioning their uses as anti-inflammatory agents. However, fenofibrate can potentially ameliorate vascular activity as we showed a decreased sE-selectin concentration. To our knowledge, there are four other studies that have compared the pharmacological intervention with fibrate and a nutritional intervention with fish oil treatment side-by-side. In hyperlipidemic T2D patients, it was shown that a greater hypolipidemic efficacy was achieved by 2-weeks treatment with the fibrate gemfibrozil (900 mg/d) compared to n-3 LCPUFAs (4.6 g/d) and that neither of the treatments affected carbohydrate metabolism⁷⁴. Another study comparing the effects of gemfibrozil (1200 mg/d) and n-3 fatty acids (3.2 g/d of EPA+DHA) in hypertriglyceridemic patients showed favorable effects of both treatments on lipid and lipoprotein concentrations and the LDL-subfraction profile⁷⁵. However, treatment with n-3 LCPUFAs did increase susceptibility of LDL to oxidation⁷⁵. In HIV-infected patients with moderate hypertriglyceridemia, 18-months of gemfibrozil treatment and n-3 LCPUFA treatment both lowered TG levels, but gemfibrozil with a higher efficacy⁷⁶. A study making the comparison between fenofibrate treatment and n-3 fatty acid treatment for 8 weeks in patients with familial dysbetalipoproteinemia (FDL) and familial hypertriglyceridemia, also included hemorrheological parameters in addition to lipid parameters⁷⁷. This study also showed that both treatments had comparable effects on lipid parameters in both patient groups, but fenofibrate exerted additional beneficial effects on fibrinogen levels and blood viscosity in FDL patients⁷⁷. In line with other studies, we showed that n-3 fatty acids from fish oil could compete with fenofibrate with respect to TG lowering in healthy overweight and obese subjects. However, pharmacological dosages of fish oil (2-4 g/d) are needed to obtain this effect²¹, which also exert unbeneficial effects on LDL cholesterol, especially in patients with hypertriglyceridemia.

3.2 Targeting PPARs: risky business?

The metabolic characteristics of PPARs and their involvement in metabolic diseases, may make it worthwhile to invest in the therapeutic potential of targeting PPAR activity. This can help in the development of new effective PPAR-based therapies to prevent or treat metabolic abnormalities associated with the metabolic syndrome. However, due to the versatile actions of PPARs, it is difficult to reach specific preventive or therapeutic effects without increasing the risk of undesirable side-effects. In our study, we measured specific enzymes and components derived from liver and kidney as safety markers. We showed that a 6-week treatment with fenofibrate in overweight and obese subjects significantly elevated alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) (**chapter 4**), although these markers remained in the normal ranges. On the contrary, fish oil did not negatively influence these markers. As already discussed, we found both beneficial and unbeneficial changes on cardiovascular

risk markers after fenofibrate and rosiglitazone treatments (**chapters 3 and 4**).

There is still a gap between pre-clinical data indicating a protective effect of PPAR ligands on cardiovascular disease risk and the effects shown by clinical trials⁷⁸. The great majority of clinical trials with fibrates as PPAR α ligands indeed indicate a reduced risk of cardiovascular events⁷⁹. The effect of thiazolidinediones, especially rosiglitazone, is heavily debated. A meta-analysis by Nissen and Wolfski in 2007 covering 42 clinical trials has shown that rosiglitazone treatment in T2D subjects was associated with a significantly increased risk of myocardial infarction and there was a trend towards an increase in fatal events with cardiovascular cause⁸⁰. After the publication, the accuracy of the methodology used for this meta-analysis was challenged⁸¹. Results of a large follow-up study on long-term cardiovascular outcome with rosiglitazone treatment (Rosiglitazone Evaluated for Cardiovascular Outcomes in Oral Agent Combination Therapy for Type 2 Diabetes (RECORD) showed inconclusive results with respect to CVD⁸². Nissen and Wolfski updated their meta-analysis, including the RECORD study, and they still showed an increased risk for myocardial infarction, but not for cardiovascular mortality⁸³. The debate concerning the safety of TZDs and rosiglitazone is still ongoing, but the European Medicine Agency's (EMA) scientific committee, the Committee for Medicinal Products for Human Use (CHMP) concluded that the benefits of rosiglitazone did not outweigh the risk and that placing these products on market for sale should be stopped⁸¹.

Synthetic PPAR agonists are also able to modulate the expression of a large number of genes, some of which produce unknown effects. It can be questioned if more selective and more powerful drugs would not result in more robust beneficial effects. This is probably a tough challenge, as two multicenter trials with a far more powerful new synthetic PPAR α agonist did not produce more favorable effects on lipid parameters compared to fenofibrate⁸⁴. The controversy that still exists around safety of synthetic PPAR agonists does not automatically imply that there is no need to look for nutritional modulators of these transcription factors for disease prevention. PPARs did not evolve merely as drug targets, but as lipid targets. A nutritional approach can provide a more subtle strategy to obtain beneficial effects with less side-effects.

4. Biomarkers for cardiovascular risk prediction

Over the last decade, many attempts have been made to predict the risk for developing CVD in individuals. The major risk factors identified included age, sex tobacco use, systolic blood pressure, need for anti-hypertensive medication use, diabetes mellitus, and LDL-cholesterol⁸⁵. Exposure to one or more of these risk factors already associate with 70-80% of CVD events⁸⁵. The Framingham Risk Score (FRS) is a good and frequently used model to estimates a person's risk of a CHD event over the next 10 years, based on an algorithm that incorporates these traditional risk factors⁸⁶. For many years now, there has been a consensus that a causal relationship exists between high levels of LDL-cholesterol with

cardiovascular diseases⁸⁷⁻⁹⁰. However, this consensus is currently a hot topic of debate, like the role of HDL-cholesterol as a risk factor for CVD⁹¹⁻⁹³.

The discovery that inflammation clearly plays a role in type 2 diabetes and CVD^{42, 44}, has opened a new field of research to establish if inflammation markers can contribute to risk assessment for CVD, which may increase the sensitivity and specificity of risk scores. We measured a wide range of inflammatory markers and markers of vascular activity in the studies described in this thesis, but changes were inconsistent. For example, rosiglitazone treatment in T2D patients reduced CRP and elevated TNF α (**chapter 3**). In overweight and obese subjects, fenofibrate treatment reduced sE-selectin and increased MCP1 (**chapter 4**). Another approach we used was to study molecular markers of immune function, such as expression the pro-inflammatory transcription factor NF κ B and its related genes, as we studied after rosiglitazone treatment in PBMCs of T2D patients (**chapter 3**). Hardly any gene changed upon treatment and the sparse molecular effects did not reflect the changes in plasma inflammatory markers. Therefore, it is very hard and probably even impossible to say how these changes in molecular and plasma immune function markers can be translated to a biologically meaning in relation to prediction of (cardiovascular) health outcome. Whole genome expression studies for discovering predictive molecular biomarkers have had modest success in the field of oncology⁹⁴, but not yet in the field of CVD. For an inflammatory biomarker to become widely accepted, it needs multiple studies in various populations showing that it predicts a part of the CVD risk that is not already captured by traditional markers, such as LDL cholesterol. And even then, the applicability for a large population and cost-effectiveness will probably limit the routine use in clinical practice. CRP is such a non-traditional candidate biomarker, which use in clinical practice is expanding⁹⁵. The addition of CRP to the FRS can provide additional prognostic information when evaluating intermediate-risk patients^{85, 96, 97}. Although more research is required⁹⁸, CRP may improve classification of subjects with intermediate risk by 12%^{97, 99}. Continuous and active research in this field will probably lead to the identification and/or validation of more markers of inflammation, vascular function and thrombosis contributing to and predicting CVD risk that is not yet captured by traditional markers.

5. Nutrigenomics research and PBMCs

Functional genomics provided a revolution in science with the sequencing of the entire human genome (Human Genome Project) in the 1990's and the development of high-throughput methods to profile genes (transcriptomics), proteins (proteomics), and metabolites (metabolomics). These techniques were incorporated into nutritional sciences, and nutrigenomics came into existence. This enabled researchers to study responses to diets or single nutrients in relation to health. Expectations of nutrigenomics were very high, predicting effective dietary intervention strategies to restore homeostasis and prevent diet-related diseases, providing a basis for personalized diets¹⁰⁰⁻¹⁰². Now in 2011, it must be

concluded that it is still in its infancy and still needs a substantial amount of time to deliver its promises. Applying microarray techniques in studies will generate huge amounts of data, since numerous genes, proteins or metabolites can be measured simultaneously. A caveat of generating enormous datasets is the difficulty in translating the data to a biological meaning, especially considering the complex inter-relationships between genes, gene regulation, proteins and metabolites.

The current status of nutrigenomics in human intervention studies has been comprehensively reviewed by Wittwer *et al.*¹⁰³. This review indicates that most published human nutrigenomics studies are descriptive of nature and show that dietary components can indeed affect genes, proteins and/or metabolites without providing a comprehensive and detailed interpretation of biological meaning. As for this thesis, interpretation difficulties were also experienced when studying huge gene expression datasets after fenofibrate and fish oil in PBMC, muscle and adipose tissue samples of overweight and obese subjects (**chapter 5**). Although sophisticated pathway analysis tools are available and used to analyze transcriptomic data, interpretation of the acquired results in relation to expression of genes and subsequent prediction of health outcomes remains very complex¹⁰⁴.

A drawback of studying whole genome expression is the limited accessibility of human tissue. This is especially the case for the metabolic syndrome, involving many tissues, like liver, muscle and adipose tissue. It has already been shown that PBMCs have an active metabolism¹⁰⁵ and respond to nutritional interventions, but it is not clear if they as well serve as good surrogate cells reflecting the condition of specific target tissues. As PBMCs also express PPARs, it was worthwhile to study if PBMCs are applicable surrogate with respect to the molecular effects of PPAR agonists (**chapter 3 and 5**). PBMCs of overweight subjects were found to respond to a nutritional (fish oil) and pharmacological intervention (fenofibrate) by changing their gene expression profile. Especially, lipid metabolic pathways were affected by fenofibrate and fish oil treatment. Reviewing literature on PBMCs as surrogate cells (**chapter 2**) indeed consistently shows the capability of PBMCs to reflect lipid metabolic genes and expression changes in these genes upon hypolipidemic dietary and pharmacological interventions (**chapter 2**). This seems not the case for inflammation, as changes in inflammation-related genes did not correspond with changes in plasma parameters, or vice versa (**chapter 3, 4, 5**). This also shows the limitation of transcriptomics that gene expression changes are not necessarily reflected in protein levels and/or protein function¹⁰³.

Despite the several physiological effects we observed after treatment with PPAR α or PPAR γ agonists, these effects were not or only very marginal reflected by an increased expression of PPAR target genes in PBMCs. This is in correspondence to another study providing 1.8 g/d of EPA/DHA for 26 weeks to healthy Dutch elderly³⁵. The lack of effect on PPAR target genes is most probably related to treatment duration, because fasting or postprandial effects of PPAR agonists on PPAR target genes are much more pronounced^{34, 36}. Results on short-term exposure may therefore be somewhat misleading, as secondary effects of

longer-term exposure can mask the direct effect on PPAR target genes. Also of relevance is that PBMCs consist of a heterogeneous cell population, with each population having its own function and its own specific transcriptome. It has also been shown that PPARs influence these distinct cell types in different ways^{45, 106}. However, the isolation of subpopulations is less feasible, as it requires a larger amount of blood and a substantial longer isolation procedure.

Transcriptomics in PBMCs is still in its infancy, especially with respect to the suitability of PBMCs reflecting other inaccessible tissues¹⁰⁷. From literature, PBMCs seems most promising in reflecting the liver on a molecular level with respect to lipid metabolic pathways (**chapter 2**). Recently, it was also reported that PBMCs express a large proportion of transcripts at comparable levels that are found in smooth muscle cells after n-3 PUFA supplementation¹⁰⁸. However, this study had no control group or baseline samples to investigate the response to n-3 PUFAs. We showed only very little overlap in gene expression changes between WAT, muscle and PBMCs (**chapter 5**). However, there might be a possible role for PBMCs as surrogate cells for WAT, due to the large resemblance in altered pathways after fish oil treatment.

Certainly, nutrigenomics brought nutritional research to a higher level, but is not yet fully exploited. Hopefully, scientists will manage to overcome existing technical and biological problems and improve evaluation and interpretation of datasets. Using a combination of complementary nutrigenomic techniques in human studies, namely transcriptomics, proteomics and metabolomics ,together with functional measurements may provide a better understanding of the complex human biological system, how this system is disturbed at the onset of disease, and how it can be modulated by nutrition.

6. Conclusions and recommendations

The studies described in this thesis show that PPAR α and γ agonists are able to exert beneficial modulate metabolic risk factors in type 2 diabetic and overweight subjects. In some instances, nutritional PPAR α agonists may provide an alternative for synthetic agonists, as we show that both fish oil as fenofibrate can exert a comparable TG lowering effect. However, the comparison does not go beyond this effect and high pharmacological dosages of fish oil are needed for this effect. Lower dosages are recommended, as it seems that the cardioprotective effects are more likely mediated via anti-arrhythmic and anti-coagulant properties. A low-grade inflammatory state is often associated with the metabolic syndrome and *in vitro* studies showed the ability of PPAR agonists to block pro-inflammatory transcription factor NF κ B. Nevertheless, rosiglitazone, fenofibrate and fish oil treatment hardly and inconsistently affected markers of inflammation in humans. PPAR agonists are able to induce gene expression changes in PBMCs, although these changes seem not to be a reflection of direct effects of PPAR activation. Furthermore, there is only very little overlap in gene expression changes between PBMCs, WAT, and muscle after fish oil and fenofibrate treatment. Nevertheless, PBMCs might have potency to serve as

surrogate cells for WAT for gene expression analysis, since they showed a large overlap in regulated pathways.

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ADDITIONAL FILES

Additional file 1 - NFκB-related genes measured with the NFκB RT²Profiler PCR array

Gene name	Gene function	Unigene code
AGT ^a	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Hs.19383
AKT1	V-akt murine thymoma viral oncogene homolog 1	Hs.525622
ATF1	Activating transcription factor 1	Hs.435267
BCL10	B-cell CLL/lymphoma 10	Hs.567382
BCL3	B-cell CLL/lymphoma 3	Hs.31210
BF	B-factor, properdin	Hs.69771
BIRC2	Baculoviral IAP repeat-containing 2	Hs.503704
CARD4	Caspase recruitment domain family, member 4	Hs.405153
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	Hs.2490
CASP8	Caspase 8, apoptosis-related cysteine peptidase	Hs.369736
CCL2	Chemokine (C-C motif) ligand 2	Hs.303649
CD40	CD40 antigen (TNF receptor superfamily member 5)	Hs.472860
CFLAR	CASP8 and FADD-like apoptosis regulator	Hs.390736
CHUK	Conserved helix-loop-helix ubiquitous kinase	Hs.198998
CSF2 ^a	Colony stimulating factor 2 (granulocyte-macrophage)	Hs.1349
CSF3 ^a	Colony stimulating factor 3 (granulocyte)	Hs.2233
SLC44A2	Solute carrier family 44, member 2	Hs.515134
EDARADD	EDAR-associated death domain	Hs.352224
EDG2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Hs.126667
EGR1 ^a	Early growth response 1	Hs.326035
ELK1 ^a	ELK1, member of ETS oncogene family	Hs.181128
F2R	Coagulation factor II (thrombin) receptor	Hs.482562
FADD	Fas (TNFRSF6)-associated via death domain	Hs.86131
FASLG	Fas ligand (TNF superfamily, member 6)	Hs.2007
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	Hs.25647
GJA1	Gap junction protein, alpha 1, 43kDa (connexin 43)	Hs.74471
HMOX1	Heme oxygenase (decycling) 1	Hs.517581
HTR2B	5-hydroxytryptamine (serotonin) receptor 2B	Hs.421649
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Hs.515126
IFNA1 ^a	Interferon, alpha 1	Hs.37026
IFNB1	Interferon, beta 1, fibroblast	Hs.93177
IFNG	Interferon, gamma	Hs.856
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	Hs.413513
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	Hs.321045
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	Hs.43505
IL10 ^a	Interleukin 10	Hs.193717
IL1A ^a	Interleukin 1, alpha	Hs.1722
IL1B	Interleukin 1, beta	Hs.126256
IL1R1	Interleukin 1 receptor, type I	Hs.557403
IL6	Interleukin 6 (interferon, beta 2)	Hs.512234
IL8	Interleukin 8	Hs.624
IRAK1	Interleukin-1 receptor-associated kinase 1	Hs.522819
IRAK2	Interleukin-1 receptor-associated kinase 2	Hs.449207
JUN	V-jun sarcoma virus 17 oncogene homolog (avian)	Hs.525704

Gene name	Gene function	Unigene code
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	Hs.36
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	Hs.1116
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	Hs.180566
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	Hs.508461
MYD88	Myeloid differentiation primary response gene (88)	Hs.82116
NALP12	NACHT, leucine rich repeat and PYD containing 12	Hs.367965
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Hs.431926
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Hs.73090
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Hs.81328
PPM1A	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	Hs.130036
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	Hs.159130
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	Hs.370620
RELA	V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	Hs.502875
RELB	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	Hs.307905
RFP2	Ret finger protein 2	Hs.436922
RHOA	Ras homolog gene family, member A	Hs.247077
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	Hs.519842
SLC20A1	Solute carrier family 20 (phosphate transporter), member 1	Hs.187946
STAT1	Signal transducer and activator of transcription 1, 91kDa	Hs.470943
TBK1	TANK-binding kinase 1	Hs.505874
TICAM2	Toll-like receptor adaptor molecule 2	Hs.278391
TLR1	Toll-like receptor 1	Hs.111805
TLR2	Toll-like receptor 2	Hs.519033
TLR3	Toll-like receptor 3	Hs.29499
TLR4	Toll-like receptor 4	Hs.174312
TLR6	Toll-like receptor 6	Hs.366986
TLR7	Toll-like receptor 7	Hs.443036
TLR8	Toll-like receptor 8	Hs.272410
TLR9	Toll-like receptor 9	Hs.87968
TMED4	Transmembrane emp24 protein transport domain containing 4	Hs.510745
TNF	Tumor necrosis factor (TNF superfamily, member 2)	Hs.241570
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	Hs.211600
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	Hs.401745
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	Hs.521456
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Hs.279594
TNFRSF7	Tumor necrosis factor receptor superfamily, member 7	Hs.355307
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	Hs.478275
TNFSF14 ^a	Tumor necrosis factor (ligand) superfamily, member 14	Hs.129708
TRADD	TNFRSF1A-associated via death domain	Hs.460996
TICAM1	Toll-like receptor adaptor molecule 1	Hs.29344

Additional file 2 List of measured PPAR α target genes

Gene	Entrez ID	Gene description
ACADL	33	acyl-Coenzyme A dehydrogenase, long chain
ACADM	34	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain
ACOX1	51	acyl-Coenzyme A oxidase 1, palmitoyl
ACOX 2	8309	acyl-Coenzyme A oxidase 2, branched chain
ACOX3	8310	acyl-Coenzyme A oxidase 3, pristanoyl
ACSL1	2180	acyl-CoA synthetase long-chain family member 1
ACSL3	2181	acyl-CoA synthetase long-chain family member 3
ACSL5	51703	acyl-CoA synthetase long-chain family member 5
ACSL6	23305	acyl-CoA synthetase long-chain family member 6
ADIPOQ	9730	adiponectin, C1Q and collagen domain containing
ANGPTL4	51120=9	angiopoietin-like 4
APOA1	335	apolipoprotein A-I
APOA2	336	apolipoprotein A-II
APOA5	116519	apolipoprotein A-V
APOC3	345	apolipoprotein C-III
AQP7	364	aquaporin 7
CD36	948	CD36 molecule (thrombospondin receptor)
CPT1A	1374	carnitine palmitoyltransferase 1A (liver)
CPT1B	1375	carnitine palmitoyltransferase 1B (muscle)
CPT1C	126129	carnitine palmitoyltransferase 1C
CPT2	1376	carnitine palmitoyltransferase II
CYP27A1	1593	cytochrome P450, family 27, subfamily A, polypeptide 1
CYP7A1	1581	cytochrome P450, family 7, subfamily A, polypeptide 1
CYP8B1	1582	cytochrome P450, family 8, subfamily B, polypeptide 1
DBI	1622	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)
EHHADH	1962	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
FABP1	2168	fatty acid binding protein 1, liver
FABP2	2169	fatty acid binding protein 2, intestinal
FABP4	2167	fatty acid binding protein 4, adipocyte
FABP6	2172	fatty acid binding protein 6, ileal (gastrotropin)
FABP7	2173	fatty acid binding protein 7, brain
FADS2	9415	fatty acid desaturase 2
GK2	2712	glycerol kinase 2
HMGCS2	3158	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)
ILK	3611	integrin-linked kinase
LPL	4023	lipoprotein lipase
NRIH3	10062	nuclear receptor subfamily 1, group H, member 3
OLR1	4973	oxidized low density lipoprotein (lectin-like) receptor 1
PCK1	5105	phosphoenolpyruvate carboxykinase 1 (soluble)
PCK2	5106	phosphoenolpyruvate carboxykinase 2 (mitochondrial)
PDPK1	5170	3-phosphoinositide dependent protein kinase-1
PLTP	5360	phospholipid transfer protein
PPARA	5465	peroxisome proliferator-activated receptor alpha
PPARD	5467	peroxisome proliferator-activated receptor delta
PPARG	5468	peroxisome proliferator-activated receptor gamma
RXRA	6256	retinoid X receptor, alpha
RXRB	6257	retinoid X receptor, beta
RXRG	6258	retinoid X receptor, gamma
SLC27A1	376497	solute carrier family 27 (fatty acid transporter), member 1
SLC27A2	11001	solute carrier family 27 (fatty acid transporter), member 2
SLC27A4	10999	solute carrier family 27 (fatty acid transporter), member 4
SLC27A5	10998	solute carrier family 27 (fatty acid transporter), member 5
SLC27A6	28965	solute carrier family 27 (fatty acid transporter), member 6
SORBS1	10580	sorbin and SH3 domain containing 1
UBC	7316	ubiquitin C
UCPI	7350	uncoupling protein 1 (mitochondrial, proton carrier)

SUMMARY

Summary

The incidence of the metabolic syndrome is rapidly increasing world-wide and predisposes to the development of type 2 diabetes (T2D) and cardiovascular diseases (CVD). Obesity is often associated with various risk factors characterizing the metabolic syndrome, such as abnormal lipid and carbohydrate metabolism and a pro-inflammatory state of the body. Peroxisome proliferator activated receptors (PPARs) α and γ are interesting molecular targets in metabolic risk management, as these receptors are involved in many metabolic pathways and present in various tissues. They can be activated by drugs and dietary fatty acids. Comparing a pharmacological and a nutritional approach to target PPARs may reveal a potential role for nutrition in modulating these transcription factors to ameliorate metabolic disturbances. Applying a nutrigenomics approach is an interesting way to explore the molecular effects of pharmacological and nutritional challenges on the metabolic state or condition of specific target tissues. However, access to specific metabolically relevant target tissues, such as muscle and adipose tissue, is complicated in humans. Therefore, it would be advantageous to be able to use the readily accessible peripheral blood mononuclear cells (PBMCs). PBMCs also express PPARs, making them of interest to study the molecular effects of these transcription factors.

The major objective of the studies described in this thesis was to examine the physiological and molecular effects of PPARs in various metabolic processes and to identify a potential role for nutritional fatty acids in modulating these transcription factors. The second objective was to evaluate the suitability of PBMCs as surrogate cells for tissues to reflect PPAR-related gene expression and gene expression in general.

Lipid, lipoprotein and glucose metabolism

PPAR α predominantly regulates lipid metabolism and effectively lowers blood triglyceride concentration. This hypotriglyceridemic effect is ascribed to a PPAR-mediated increase in activity of the enzyme lipoprotein lipase (LPL) and subsequent breakdown of triglycerides. In our 6-week intervention with fish oil (3.7 g/d n-3 long chain polyunsaturated fatty acids) and fenofibrate (200 mg/d) in overweight and obese subjects, we indeed found strong hypotriglyceridemic effects for both treatments. This was accompanied by reductions in large very low density lipoprotein (VLDL) particles. Fenofibrate further improved the serum lipid profile, as also low density lipoprotein (LDL) cholesterol concentrations decreased and high density lipoprotein (HDL) cholesterol increased. In contrast to fenofibrate, fish oil increased LDL cholesterol concentrations. For fish oil and fenofibrate treatment, it was expected that the LDL particle size would change to a more buoyant and less atherogenic type, but we could not confirm this in our study. Neither fenofibrate nor fish oil exerted effects on plasma glucose concentrations.

PPAR γ is mainly known for its insulin-sensitizing effects, which is related to an increased glucose uptake and stimulation of adipocyte differentiation to create more smaller and insulin sensitive adipocytes. Indeed, our intervention study of an 8-week treatment with the synthetic PPAR γ agonist rosiglitazone (8 mg/d) improved insulin sensitivity in type 2 diabetic patients, making these patients as insulin sensitive as healthy reference subjects. Furthermore, rosiglitazone also improved the serum lipid

profile, as shown by reduced serum triglycerides and free fatty acids and by increased HDL cholesterol concentrations.

Markers of inflammation and vascular activity

The metabolic syndrome is also characterized by a state of low-grade inflammation, although this is not part of its official diagnostic criteria. Since inflammation has been implicated in the development of type 2 diabetes and cardiovascular diseases, it is of relevance to prevent this. It has been reported that PPAR α and γ agonists are able to inhibit proinflammatory transcription factors, such as nuclear factor κ B (NF κ B). The cardioprotective effect of fish oil has also been partly described to a reduction in markers of low-grade inflammation. In our mechanistic in vitro study in human liver (HepG2) cells, we showed that fenofibrate and different fatty acids were indeed able to inhibit NF κ B activation, although the effects of fatty acids seemed to be largely independent of PPAR α . Despite this finding, we did not observe potent anti-inflammatory effects of PPAR agonists that can be linked with CVD reduction in our human intervention studies. Only very few plasma inflammatory and vascular activity markers were, inconsistently, affected by rosiglitazone, fenofibrate and fish oil, showing both pro- and anti-inflammatory effects. Rosiglitazone decreased macrophage chemoattractant protein 1 (MCP1) and C-reactive protein (CRP), but increased tumor necrosis factor α (TNF α) in type 2 diabetic patients. Fenofibrate reduced soluble endothelial selectin (sE-selectin), but increased MCP1 in overweight and obese subjects and no changes were seen upon fish oil treatment.

Gene expression

PBMCs can be seen as sentinels that travel through the body and pick up physiological signals. It has been shown that PBMCs have an active metabolism, express PPARs and are able to respond to nutritional interventions. However, it is not clear if these cells are adequate surrogate cells reflecting the condition of relatively inaccessible target tissues. Reviewing the literature on this topic showed that PBMCs have the capability to reflect lipid metabolic genes and expression changes in these genes upon dietary or pharmacological hypolipidemic treatment. PBMCs could therefore potentially be surrogate cells to reflect the liver with respect to lipid metabolism. We performed whole genome microarray analysis on PBMCs, muscle and white adipose tissue (WAT) samples from eleven subjects who received 6-week treatments with placebo, fish oil (3.7 g/d n-3LCPUFA) and fenofibrate (200 mg/d). These tissues are relevant in the etiology of the metabolic syndrome and could reveal important (PPAR α -regulated) genes implicated in metabolic risk reduction. Despite the several physiological effects we observed after PPAR α or γ agonists in our intervention studies, these effects were not, or only very marginally, reflected by changes in (PPAR-) related genes. Especially, lipid metabolic gene expression pathways were downregulated after fish oil and fenofibrate treatment in PBMCs. In addition, mitochondrial metabolic pathways were downregulated in both treatments, which may be related to peroxisome proliferation. Fenofibrate hardly affected gene expression in muscle and adipose tissue, showing that these tissues are not primary

PPAR α target tissues. There was hardly any overlap in gene expression changes between all three tissues. However, there might be a role for PBMCs to serve as surrogate for adipose tissue, due to the large resemblance in altered pathways upon fish oil treatment. These pathways were mainly involved in lipid and amino acid metabolism.

Conclusion

The studies described in this thesis show that PPAR α and γ agonists are able to beneficially modulate metabolic risk factors in type 2 diabetic and overweight subjects. In some instances, nutritional PPAR α agonists may provide an alternative for synthetic agonists, as we show that both fish oil as fenofibrate can exert a comparable TG lowering effect. However, the comparison does not go beyond this and high dosages of fish oil are needed to obtain this effect. Despite their in vitro capability to block pro-inflammatory transcription factor NF κ B, the PPAR agonists rosiglitazone, fenofibrate and fish oil inconsistently affected markers of inflammation in humans, if at all. The studies in this thesis also show that PPAR agonists are able to induce gene expression changes in PBMCs, although these changes seem not to be a reflection of direct effects of PPAR activation. Despite the very little overlap in gene expression changes between PBMCs, WAT, and muscle after fish oil and fenofibrate treatment, PBMCs might have potency to serve as surrogate cells for WAT for gene expression analysis, since these two cell types showed a large overlap in regulated pathways after fish oil treatment.

SAMENVATTING

Samenvatting

De incidentie van het metabool syndroom neemt wereldwijd snel toe en vergroot het risico op het ontwikkelen van type 2 diabetes (T2D) en hart- en vaatziekten (HVZ). Overgewicht en obesitas zijn vaak geassocieerd met verschillende risicofactoren die het metabool syndroom karakteriseren, waaronder een abnormaal vet- en koolhydraatmetabolisme en een laag-grade ontstekingsstatus van het lichaam. Peroxisoom prolifererence receptoren (PPARs) α en γ zijn interessante moleculaire doelwitten voor management van metabole risico's, omdat deze receptoren bij veel metabole processen betrokken zijn en in diverse weefsels tot expressie komen. Ze kunnen geactiveerd worden door medicijnen en vetzuren uit de voeding. Door een vergelijking te maken tussen een farmacologische en voedingskundige benadering om PPARs te beïnvloeden, kan een mogelijke rol van voeding ontrafeld worden ter voorkoming van metabole verstoringen. Een interessante manier om de moleculaire effecten van een farmacologische en voedingsinterventie op de metabole conditie van specifieke weefsels, zoals vet- en spierweefsel, te onderzoeken is door middel van nutrigenomics. Echter, deze specifieke weefsels zijn in mensen moeilijk beschikbaar voor onderzoek. Het zou daarom gunstig zijn als de eenvoudig bereikbare perifere mononucleaire bloedcellen (PBMC) hiervoor gebruikt zouden kunnen worden. Deze bloedcellen brengen immers ook PPARs tot expressie, waardoor ze interessante surrogaatcellen kunnen zijn om de moleculaire effecten van deze transcriptiefactoren te onderzoeken.

De hoofddoelstelling van de studies beschreven in dit proefschrift was om de fysiologische en moleculaire effecten te onderzoeken van PPARs in diverse metabole processen en om daarbij de rol van vetzuren uit de voeding te identificeren voor het moduleren van deze transcriptiefactoren. De secundaire doelstelling was om de geschiktheid van PBMCs te evalueren als surrogaatcellen voor andere lichaamsweefsels om (PPAR-gerelateerde) genexpressie te reflecteren.

Lipide-, lipoproteïne- en glucosemetabolisme

PPAR α reguleert voornamelijk het vetmetabolisme en activatie van deze receptor leidt tot verlaging van de triglyceride concentratie in het bloed. Dit hypotryglyceridemische effect is toe te schrijven aan een toename van lipoproteïne lipase activiteit via PPAR α . In onze zesweekse interventie met visolie (3.7 g/d n-3 lange keten meervoudig onverzadigde vetzuren (n-3 LCPUFA)) en fenofibraat (200 mg/d) in mensen met overgewicht en obesitas vonden we inderdaad sterke triglyceride-verlagende effecten na beide interventies. Dit ging samen met verlagingen in VLDL vetdeeltjes. Fenofibraat verbeterde het serumvetprofiel effectief, door ook een verlaging van het LDL cholesterol en verhoging van het HDL cholesterol te bewerkstelligen. Dit in tegenstelling tot visolie, waarbij een stijging van het LDL cholesterol werd waargenomen. Er werd verwacht dat de visolie- en fenofibraatinterventie de LDL deeltjes groter zouden worden met een lagere dichtheid, waardoor ook minder atherogeen. Dit konden we met onze studie echter niet bevestigen. Daarnaast leidde een interventie met fenofibraat of visolie niet tot veranderingen in plasmaglucoseconcentraties.

PPAR γ staat bekend om het insuline-gevoeliger maken van het lichaam,

wat gerelateerd is aan een toename van glucoseopname en een stimulering van vetcelldifferentiatie, waarbij meer kleine, insulinegevoeligere vetcellen worden gevormd. Inderdaad liet onze achttweekse interventie met de synthetische PPAR γ agonist rosiglitazone zien dat de insuline-gevoeligheid toenam in patiënten met type 2 diabetes. Deze mensen werden zelfs weer net zo insulinegevoelig als de gezonde personen die als referentiegroep dienden. Verder verbeterde rosiglitazone ook het serumvetprofiel, door triglyceriden en vrije vetzuurconcentraties te verlagen en door HDL-cholesterolconcentraties te verhogen.

Markers voor laag-grade ontsteking en vasculaire activiteit

Het metabool wordt ook gekarakteriseerd door een staat van laag-grade ontsteking, al hoort dit niet tot de officiële criteria om het metabool syndroom te diagnosticeren. Het is belangrijk dit te voorkomen, omdat laag-grade ontsteking betrokken is bij het ontstaan van T2D en HVZ. Van PPAR α en γ activatoren is gerapporteerd dat ze pro-inflammatoire transcriptiefactoren kunnen remmen, zoals de transcriptiefactor nucleaire factor kappa bèta (NF κ B). De beschermende werking van visolie op het ontstaan van HVZ wordt ook gedeeltelijk toegeschreven aan de verlaging van laag-grade ontstekingsmarkers. In onze mechanistische in vitro studie in humane levercellen (HepG2), laten we zien dat fenofibraat en verschillende vetzuren de transcriptiefactor NF κ B inderdaad kunnen remmen, al leek dit effect van vetzuren grotendeels onafhankelijk van PPAR α te zijn. Ondanks deze observatie zagen we in onze humane studie geen sterke ontstekingsremmende effecten van PPAR agonisten die gelinkt zouden kunnen worden aan een verminderd risico op het ontwikkelen van HVZ. Zeer weinig laag-grade ontstekingsmarkers en markers van vasculaire activiteit werden beïnvloed door rosiglitazone, fenofibraat en visolie. De veranderingen die werden waargenomen waren ook inconsistent, dus zowel mogelijk anti- als pro-inflammatoire effecten. Rosiglitazone zorgde er voor dat de concentraties van monocyt chemotactisch eiwit 1 (MCP1) en C-reefief proteïne (CRP) daalden, maar deed de concentratie van tumor necrosefactor α (TNF α) stijgen in patiënten met T2D. Door fenofibraat daalde oplosbaar endotheliale selectine (E-selectin), maar het deed MCP1 stijgen in mensen met overgewicht en obesitas. Door visolie veranderde zelfs geen van de markers voor laag-grade ontsteking en vasculaire activiteit.

Genexpressie

PBMCs kunnen gezien worden als schildwachten die door het lichaam surveilleren en fysiologische signalen kunnen oppikken. Het is gerapporteerd dat PBMCs een actief metabolisme hebben, PPARs tot expressie brengen en reageren op voedingsinterventies. Echter, het is niet duidelijk of deze cellen goede surrogaatcellen zijn om de status van relatief onbereikbare doelweefsels weer te geven. De literatuur over dit onderwerp laat zien dat PBMCs de eigenschap bezitten om genen betrokken bij het vetmetabolisme te reflecteren en de expressie van deze genen te laten veranderen op een vetverlagende farmacologische of voedingsinterventie. PBMCs zouden om deze reden interessante surrogaatcellen kunnen vormen voor de lever wat betreft vetmetabolisme. We

hebben microarray-analyse gedaan op het volledige genoom van PBMCs, spier- en vetweefselmonsters van elf proefpersonen die zesweekse behandelingen ondergingen met placebo, visolie (3.7 g/d n-3 LCPUFA) en fenofibraat (200 mg/d) capsules. De onderzochte weefsels zijn relevant in de etiologie van het metabool syndroom en kunnen belangrijke (PPAR α -gereguleerde) genen onthullen die betrokken zijn bij risicoverlaging.

Ondanks de verschillende fysiologische effecten na behandeling met PPAR α en γ agonisten in onze interventiestudies, werden deze effecten, als ze al optraden, maar marginaal gereflecteerd door veranderingen in (PPAR-) gerelateerde genen. In PBMCs werden voornamelijk genexpressieroutes betrokken bij vetmetabolisme naar beneden gereguleerd als reactie op visolie en fenofibraatbehandeling. Ook mitochondriale metabole routes werden naar beneden gereguleerd, wat te maken kan hebben met een toegenomen peroxisoomproliferatie. Fenofibraat had maar erg marginaal invloed op genexpressie in spier- en vetweefsel, wat laat zien dat deze weefsels geen primaire doelweefsels zijn voor PPAR α . Er was haast geen overlap in genexpressieveranderingen tussen alle drie de weefsels. Echter, PBMCs zouden een rol kunnen spelen als surrogaatcellen voor vetweefsel, omdat er een grote overeenkomst was in veranderde genexpressieroutes na visoliebehandeling. Deze routes waren met name betrokken bij lipide en aminozuurmetabolisme.

Conclusion

De studies die in dit proefschrift worden beschreven, laten zien dat PPAR α en γ agonisten in staat zijn om metabole risicofactoren gunstig te beïnvloeden in mensen met type 2 diabetes en mensen met overgewicht. In sommige gevallen zou een natuurlijke PPAR α agonist een alternatief kunnen zijn voor een synthetische agonist, omdat we hebben laten zien dat zowel visolie- als fenofibraatbehandeling een vergelijkbaar triglyceride-verlagend effect kan bewerkstelligen. Echter, de vergelijking gaat niet verder dan dit overeenkomstige effect en er is een hoge dosering visolie nodig om dit effect te bereiken. Ondanks de eigenschap van PPAR agonisten rosiglitazone, fenofibraat en visolie om in vitro de pro-inflammatoire transcriptiefactor NF κ B af te remmen, wordt dit nauwelijks en inconsistent gereflecteerd in veranderingen van plasma-ontstekingsmarkers in mensen. De studies beschreven in dit proefschrift laten ook zien dat PPAR agonisten genexpressieveranderingen in PBMCs kunnen induceren, al lijken deze veranderingen niet het directe resultaat te zijn van PPAR activering. Ondanks de zeer marginale overlap in genexpressieveranderingen na PBMCs, vetweefsel en spier na visolie en fenofibraat behandeling, zouden PBMCs wel de potentie kunnen hebben om als surrogaatcellen voor vetweefsel te dienen, omdat deze twee celtypen grote overlap in gereguleerde routes laten zien na visoliebehandeling.

DANKWOORD

PUBLICATIONS

CURRICULUM VITAE

Dankwoord

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Curriculum vitae

Marjolijn Bragt was born on January 10 1981 in Roosendaal, the Netherlands. In 1999, she completed her secondary school at "Norbertus College" in Roosendaal. Subsequently, she started her academic education and studied Health Sciences with a specialization in Biological Health Sciences and molecular biology at the Faculty of Health Medicine and Life Sciences of Maastricht University. Her internship was spent at the department of Human Biology, Maastricht University to study the effects of plant sterols and stanols on inflammation and endothelial function in statin-treated patients. She graduated in 2003. Her master's thesis was awarded with the Unilever Research Prize 2004. In October 2003, Marjolijn was appointed as a research assistant at the department of Human Biology, Maastricht University to study the effects of policosanols on ApoA1 expression in liver and intestinal cells. From September 2004 to December 2008, she worked as a PhD-fellow in the same department on a project from Top Institute Food & Nutrition under supervision of prof. dr. ir. Ronald P. Mensink. In this research project, she studied the role of peroxisome-proliferator-activated receptors (PPARs) in metabolic risk management, using a pharmacological and nutritional approach. Since September 2009, Marjolijn works as a nutritionist in the field of immunity at FrieslandCampina Innovation Infant & Toddler Nutrition in Leeuwarden, the Netherlands.