Dietary fat and the prevention of type 2 diabetes;

impact on inflammation and underlying mechanisms

Susan van Dijk

Thesis committee

Thesis supervisor
Prof. Dr. Michael Müller
Professor of Nutrition, Metabolism and Genomics
Wageningen University

Thesis co-supervisors
Prof. Dr. Edith JM Feskens
Professor of Nutrition and Metabolic Syndrome
Wageningen University

Dr. Lydia A Afman
Assistant professor Division of Human Nutrition
Wageningen University

Other members
Prof. Dr. Ivonne MCM Rietjens
Wageningen University

Prof. Dr. Hannelore Daniel
Technical University Munich, Germany

Prof. Dr. Ellen E Blaak
Maastricht University

Dr. Ben van Ommen
TNO Quality of life, Zeist

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Thesis

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Susan J van Dijk

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ABSTRACT

The incidence of metabolic syndrome, which is a risk factor for cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) is increasing rapidly. Changes in dietary fat composition from saturated fat (SFA) to monounsaturated fat (MUFA) from olive oil, which is highly consumed in a Mediterranean diet, might improve risk factors for CVD and T2DM. However, the underlying molecular mechanisms for these beneficial health effects are not completely known. Moreover, more knowledge is needed about health status and biomarkers that allow early detection of onset of diseases such as metabolic syndrome, CVD and T2DM.

The aim of this thesis was twofold; first, to investigate the acute and longer-term effects of intake of different types of dietary fat. Second, to examine whether a more comprehensive phenotyping of health status can be achieved by application of nutrigenomics tools and challenges tests.

A controlled dietary intervention study was performed in healthy abdominally overweight subjects to investigate the effects of 8-weeks consumption of diets high in SFA or MUFA on insulin sensitivity, serum lipids and adipose tissue whole genome gene expression. Moreover, the effects of replacement of SFA by MUFA, as part of a western-type diet and as part of a Mediterranean diet, on peripheral blood mononuclear cell (PBMC) whole genome gene expression and plasma protein levels were investigated.
Plasma protein profiles of the subjects before the intervention were used to define proteins and protein clusters that were associated with BMI and insulin concentrations. Similar analyses were performed in a second overweight population to verify the findings.
In two other studies, the response capacity of subjects with different metabolic risk phenotypes to a high-fat challenge varying in fat type and to an extreme caloric restriction challenge were determined.

Results from our first study showed that consumption of a SFA-rich diet increased expression of inflammation-related genes in adipose tissue whereas consumption of a MUFA-rich diet led to a more anti-inflammatory gene expression profile, without changes in insulin sensitivity or increases in body weight. Moreover, high MUFA intake from olive oil, both in a western-type diet and in a Mediterranean-type diet, lowered expression of genes involved in oxidative phosphorylation in PBMCs and

lowered serum LDL and plasma ApoB, Connective Tissue Growth Factor and myoglobin concentrations.
In plasma of the healthy participants clusters of proteins associated with BMI or insulin could be identified. These clusters included previously reported biomarkers for disease and potential new biomarkers.
The high-fat challenge study showed that the plasma metabolic response and the PBMC gene expression response to high-fat challenges were affected by the presence of obesity and/or diabetes. Comparison of responses to high SFA, MUFA and n-3 PUFA loads showed that a high MUFA load induced the most pronounced response.
The caloric restriction study revealed that PBMC gene expression profiles were different between metabolic syndrome subjects and healthy subjects, mainly for genes involved in pathways related to mitochondrial energy metabolism. Moreover, we observed that the caloric restriction challenge magnified differences in PBMC gene expression profiles between the subject groups.

In conclusion, this thesis showed that 8-weeks consumption of a SFA-rich diet resulted in a pro-inflammatory gene expression profile in adipose tissue whereas consumption of a MUFA-rich diet caused a more anti-inflammatory profile, in addition to reductions in LDL cholesterol, some plasma proteins and expression of oxidative phosphorylation genes in PBMCs. Since the effects of the diets on inflammation were still local and not accompanied by systemic changes in inflammatory status or insulin sensitivity we hypothesize that adipose tissue could be an early response organ for dietary fat-induced changes. The changes in pro-inflammatory gene expression might be one of the first hallmarks in the development of adipose tissue inflammation and insulin resistance which on the longer term may lead to inflammation-related diseases such as metabolic syndrome.
Our studies showed the potential of whole genome expression profiling, plasma profiling and the use of challenges tests to detect subtle diet effects and small differences in health status. Using these tools in future studies will result in more knowledge about health status and about mechanisms behind dietary effects. Eventually this might lead to earlier detection of small deviations from a healthy phenotype and to evidence-based dietary advice to improve health and to prevent disease.

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CHAPTER 1

General introduction

Metabolic syndrome

The incidence of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) is increasing rapidly and accounts for a substantial proportion of health care costs. Moreover, these chronic diseases represent a major cause of death in western society. The presence of the metabolic syndrome is a risk for these diseases [1, 2]. The term metabolic syndrome, or insulin resistance syndrome, was already mentioned in the 1980s as a concept to describe a group of metabolic risk factors that often cluster together [3]. The exact definition of the syndrome has been subject to changes since then and is still slightly different for each health authority. The National Cholesterol Education Program's Adult Treatment Panel III defined the metabolic syndrome as a cluster of at least three or more of the following metabolic risk factors; central obesity (waist circumference ≥102 cm for men and ≥88 cm for women), elevated fasting glucose (≥ 5.6 mmol/L), elevated triglycerides (≥ 1.7 mmol/L), reduced HDL cholesterol (≤1.03 mmol/L for men and ≤ 1.29 mmol/L for women) and hypertension (≥ 130/85 mmHg) [4, 5]. In order to improve these risk factors changes in lifestyle are recommended as an early intervention approach and dietary interventions should have a major role in this.

Dietary fat and the prevention of diabetes

Several epidemiological and observational studies showed that a high amount of fat in the diet could be important for the risk to develop diabetes and CVD. However, besides the amount of fat in the diet, the type of fat in the diet might also influence metabolic processes in the development of disease [6]. Adherence to a traditional Mediterranean type of diet, which is relatively high in fat (± 35 en%), is associated with lower CVD mortality [7, 8] and is inversely associated with features of the metabolic syndrome [9, 10]. Several health promoting effects of the Mediterranean diet have been ascribed to its high olive oil content, although other characteristics of the diet (e.g. abundant use of fruits, vegetables, cereals, legumes, nuts, seeds, moderate intake of fish and red wine and low intake of dairy products, and meat) [8, 11, 12] might also contribute to the health effects.

A Mediterranean diet contains a high percentage (16-30%) of monounsaturated fat (MUFA) from olive oil whereas a western-type diet contains a relatively high percentage of saturated fat (SFA) from butter, cheese and meat. Dietary intervention studies have shown that replacement of SFA by MUFA in the diet can reduce total cholesterol, LDL cholesterol and triglyceride concentrations [13, 14]. However, whether replacement of SFA with MUFA could also improve insulin sensitivity is still controversial [15]. Some intervention studies [16, 17] showed that insulin sensitivity increased after replacing SFA with MUFA but results from two recent multicenter

studies [18, 19] did not provide evidence that the replacement of SFA with MUFA could ameliorate insulin sensitivity.

The exact mechanisms underlying the possible association between fatty acid intake and insulin sensitivity are not completely clear but different mechanisms have been proposed. A traditional idea how fatty acids could affect insulin resistance is via alterations in cell membrane composition which can change membrane fluidity. This might affect insulin action through changes in ion permeability, cell signalling or altered insulin receptor binding and affinity. A higher desaturation and fluidity of the phospholipids in the muscle cell membrane was found to be associated with higher insulin sensitivity [16, 20].

Overconsumption of dietary fatty acids, especially SFA, can also result in accumulation of fatty acid intermediates (e.g. diacylglycerol and ceramide) in skeletal muscle, liver and adipose tissue, which can lead to activation of several serine kinases such as protein kinase Cθ (PKCθ), c-Jun N-terminal kinase (JNK1), IκB kinase β (IKKβ) and in this way negatively regulate insulin action [21, 22].

From the molecular point of view, dietary fatty acids are considered as dietary signals that can affect metabolism by activating transcription factors and thereby influencing gene expression [23]. Dietary fatty acids can have distinct effects on gene expression depending on their molecular structure. Small changes in structure (e.g. from saturated to unsaturated) can influence gene expression and the processes that are activated. Consumption of more unsaturated fatty acids instead of SFA might change the activity of specific fatty acid-regulated transcription factors, such as peroxisome proliferator-activated receptors (PPARs), Sterol Regulatory Element-Binding Proteins (SREBP), liver X receptor and NF kappa B, thereby affecting lipid, carbohydrate, and protein metabolism, as well as immune processes and cell growth [24, 25]. SFA on the other hand, may activate toll-like receptors (TLRs) of the innate immune response, which lead to activation of the NFκB and JNK pathway and could contribute to insulin resistance [26, 27].

Dietary fatty acids are mainly stored in white adipose tissue but in situations of chronic positive energy intake and decreased capacity and expandability of the subcutaneous adipose tissue depot fatty acids can also accumulate in other tissues such as skeletal muscle, liver, heart, and pancreatic β cells. Accumulation of fat in these tissues might impair tissue functioning and can result in tissue insulin resistance [21, 28]. The total amount of intracellular lipids is influenced by the balance between the rate of fatty acid oxidation and triglyceride synthesis. Dietary fatty acids may affect this intracellular lipid balance since different fatty acids were found to have different oxidation rates [29, 30].

Insulin sensitivity and adipose tissue inflammation

During the last decades, evidence has been accumulating suggesting a link between insulin resistance and chronic low-grade inflammation in adipose tissue. Adipose tissue has not only a function in energy storage but also acts as an endocrine organ by producing and secreting hormones and adipokines needed for glucose homeostasis, energy metabolism, food intake and body weight regulation, hemostasis, and immune function [28, 31]. A good balance of secretion of pro- and anti-inflammatory adipokines is essential for optimal body functioning but this balance can get disturbed when adipose tissue mass is increasing, as in subjects with obesity or metabolic syndrome. Increased adipose tissue mass can lead to changes in adipose tissue morphology, characterized by adipocyte hypertrophy, hypoxia, adipocyte death and infiltration of inflammatory cells such as macrophages and T-cells [32-35]. This results in increased production and secretion of a wide range of pro-inflammatory molecules, mainly from the non-adipocyte fraction of adipose tissue, and in decreased secretion of important metabolic regulatory molecules such as adiponectin [36, 37]. Secretion of these pro-inflammatory molecules into the bloodstream can affect the metabolic function of other organs and may impair insulin signalling in peripheral tissues, thereby causing whole body insulin resistance (figure 1) [38, 39].

The immune system and the metabolic system are highly integrated, driven by the evolutionary need to withstand starvation and to elicit an affective immune response to infectious challenges [33]. Due to the close integration of the immune and metabolic system disturbances in the metabolic system can have impact on the immune system and vice versa. Nowadays, obesity is considered as a state of chronic low-grade inflammation and metabolic diseases such as T2DM and CVD also have a large inflammatory component [40].

Several dietary factors were found to affect inflammation [41-43]. SFA was suggested to have pro-inflammatory effects, mainly based on observational and in vitro studies [22, 44-47]. A Mediterranean dietary pattern was found to reduce circulating inflammatory proteins and was considered as anti-inflammatory [48, 49].

Extensive research has been done investigating the effect of obesity or weight loss interventions on inflammatory processes in adipose tissue [50-53]. However, the impact of consumption of different dietary fatty acids, without weight loss, on processes in the adipose tissue is less well explored and needs further attention.

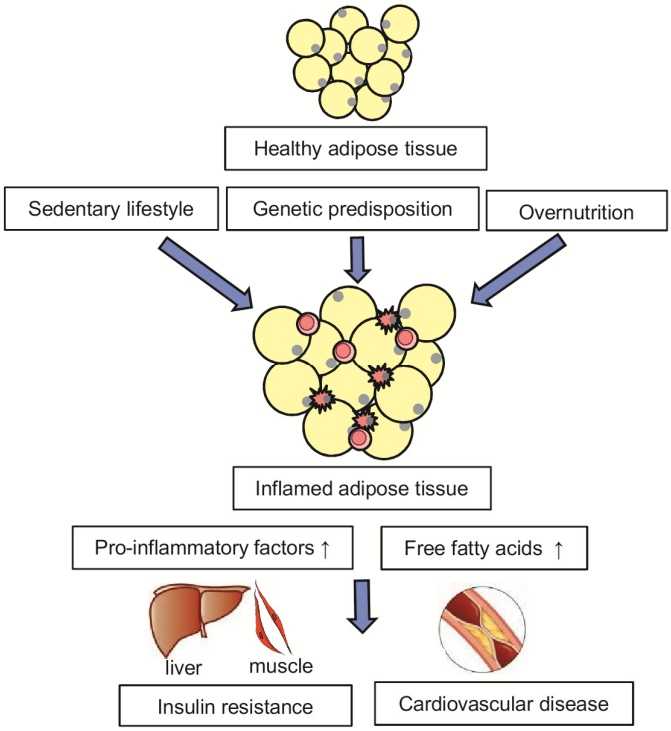

Figure 1 Increases in adipose tissue mass leading to insulin resistance and cardiovascular disease

Although diet may affect several processes related to inflammation and insulin sensitivity traditionally only a few physiological endpoints, such as plasma insulin, glucose and some inflammatory cytokines, were measured in dietary intervention studies. This made it difficult to unravel the underlying mechanisms behind the health effects of specific diets or dietary components and therefore a more comprehensive approach is necessary.

Nutrigenomics

Nutrigenomics research will allow to get more knowledge on these underlying mechanisms since it investigates the genome-wide influences of nutrition on the transcriptome, proteome and metabolome of cells, tissues or organisms [23, 54]. The transcriptome reflects the genes that are being expressed and transcriptomics examines the expression level of messenger RNA in a cell population or tissue at any given time and under various conditions. Transcriptomics uses high-throughput tools such as

whole genome DNA microarrays which enables an almost unbiased comprehensive analysis of changes in the expression of all genes in case-control or control-intervention samples. The use of transcriptomics gives insights in the genome-wide effects of specific diets or dietary components and in this way will increase understanding about the effect of nutrition on metabolic processes [54].

Peripheral Blood Mononuclear Cells

The application of transcriptomics in nutrition research is very promising but there are some restrictions for the use in human studies. For transcriptomics tissue samples are needed, but the availability of tissues from human subjects participating in dietary intervention studies is usually limited due to practical and ethical reasons. Muscle and adipose tissue biopsies are still relatively easy to collect and therefore the most sampled biopsies in human intervention studies. Theoretically it is possible to collect biopsies from other tissues such as intestine, prostate or liver but these have to be obtained through more invasive procedures which are usually not performed in healthy subjects in dietary intervention trials. Since tissue availability is a primary limitation several studies have investigated the use of human peripheral blood cells for human nutrigenomics research.

Peripheral blood mononuclear cells (PBMCs), a mixture of T-cells, B-cells and monocytes, are easily obtainable by venipuncture and are an interesting source of biological material. In the first place because PBMCs have been shown to be metabolically active but also because it is assumed that PBMCs might reflect physiological and pathological processes in different body tissues since they circulate throughout the whole body [55]. Moreover, PBMCs also play a role in inflammatory pathways that lead to atherosclerosis. Gene expression profiles of PBMCs were shown to reflect diseases states [56-58] and changes in diet composition [24, 59] and thus might serve as a suitable tool for nutrigenomics research.

Biomarkers of health

Nutrition research nowadays mainly focuses on prevention of metabolic disease and improvement of the health of individuals through diet. In order to achieve the highest benefit of nutrition subjects at risk to develop disease have to be identified as early as possible. To identify these subjects there is a need for very early biomarkers of disease to measure early signs of deviations from healthy metabolism. While currently only several single markers for disease are identified, new genomic techniques might help to define more extensive marker profiles of health and early disease state, both at transcriptome and proteome level. A marker profile, compared to single markers, might provide additional information on different and not always overlapping phases of

disease development. Especially in multifactorial, complex diseases such as T2DM and CVD this multi-marker approach is expected to be very suitable for early risk assessment [60].

Nutritional challenge

Health status is often determined on the basis of metabolic measures, such as glucose, insulin, cholesterol, or triglycerides in the fasting state. Fasting metabolic measures are rather stable and not largely influenced by preceding meal intake. Therefore it is easier to set reference values for health status to which individual values can be compared. However, individuals spent the largest part of the day in the non-fasting condition and during a day their metabolic system has to cope with successive food intake. Every meal elicits a metabolic and inflammatory stress response and the body needs to respond to maintain homeostasis. The ability of subjects to respond to nutritional challenges can reflect the robustness and flexibility of their biological system and might give a better indication of health and disease risk than fasting measures [61, 62].
Well-known examples of nutritional challenge tests are the oral fat loading test which can be used to detect impairments in lipid handling and the oral glucose tolerance test (OGTT) that is widely used to identify subjects with diabetes. The OGTT is used for diagnostic purposes because it gives an indication of the glucose clearance capacity of the body and it will enable the identification of impaired glucose tolerant subjects which cannot be recognized based on fasting glucose measurements [63].

Body fat distribution

Metabolic health can be impaired by increased adipose tissue mass as in the case in obesity. Recent studies have shown that it is not necessarily the amount of extra adipose tissue, but more importantly, the place where excess fat is stored that determines the metabolic health of a subject. The general idea is that as long as the subcutaneous adipose tissue depot has the capacity to store fat properly, adverse health effects of extra adipose tissue are limited [28]. However, when energy intake exceeds the storage capacity of subcutaneous adipose tissue, fat is deposited as visceral fat in the abdominal area and accumulates in ectopic depots such as liver [64]. Visceral adipose tissue appears to play an important role in the development of metabolic syndrome, diabetes and CVD [65, 66], but the underlying mechanisms are not completely clear. It might be because visceral fat is metabolically more active compared with subcutaneous fat. Moreover, because of its location the visceral adipose tissue can release its metabolic products such as free fatty acids into the portal vein, which provides direct delivery to the liver and might thereby promote liver insulin resistance.

Waist circumference has often been used as a surrogate measure of abdominal fat, but this measure cannot distinguish the amount of subcutaneous and visceral abdominal fat. For the measurement of the different abdominal fat depots magnetic resonance imaging (MRI) has been shown to be a valid method [67].

OUTLINE THESIS

In this thesis the acute and longer-term effects of intake of different types of dietary fat were investigated in subjects with different metabolic health profiles. To examine responses in adipose tissue, PBMCs and plasma nutrigenomics tools were used.
First, an 8-weeks completely controlled dietary intervention study was performed to investigate the effects of consuming a high saturated (SFA) or high monounsaturated (MUFA) fat diet on insulin sensitivity and adipose tissue gene expression in abdominally overweight subjects **(chapter 2).**
In the same dietary intervention study the effects of MUFA as part of a western-type diet and the effects of MUFA as part of a full Mediterranean (MED) diet on PBMC gene expression and plasma protein levels were investigated **(chapter 3).**
The abdominally overweight subjects that participated in the intervention study were healthy but at risk to develop metabolic syndrome and related diseases because of their age and abdominal overweight. In these subjects protein profiling was used to improve the phenotypic characterization of these subjects and to identify new candidate profiles for early biomarkers of obesity-related diseases such as CVD and T2DM **(chapter 4)**.
In a second study the metabolic, immune and PBMC response to high-fat challenges in subjects with different metabolic risk phenotypes (i.e. lean healthy, obese healthy and obese diabetic) were characterized and the responses to different types of fat (i.e. SFA, MUFA and n-3 PUFA) were compared. The effect of abdominal body fat distribution on the response to a high-fat challenge was also examined (**chapter 5**).
In the last study the effects of an extreme caloric restriction challenge on PBMC gene expression profiles in healthy subjects and subjects with metabolic syndrome were measured **(chapter 6)**.
In **chapter 7** the results described in the previous chapters are discussed and evaluated.

CHAPTER 2

A saturated fatty acid–rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome

SJ van Dijk, EJM Feskens, MB Bos, DWM Hoelen, R Heijligenberg, M Grootte Bromhaar, LCPGM de Groot, JHM de Vries, M Müller and LA Afman

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ABSTRACT

Background: Changes in dietary fat composition could reduce the risk for developing metabolic syndrome. The adipose tissue is an interesting tissue in this respect because of its role in lipid metabolism and inflammation.

Objective: Our objective was to investigate the effect of a saturated fatty acid (SFA)- and a monounsaturated fatty acid (MUFA)-rich diet on insulin sensitivity, serum lipids, and gene expression profiles of adipose tissue of subjects at risk for metabolic syndrome.

Design: A parallel controlled-feeding trial was conducted in 20 abdominally overweight subjects. Subjects received an SFA diet or an MUFA diet for 8 wk. Plasma and subcutaneous adipose tissue samples were obtained, and insulin sensitivity was measured by using a hyperinsulinemic-euglycemic clamp. Adipose tissue samples underwent whole genome microarray histologic analysis. Plasma and adipose tissue fatty acid composition and concentrations of serum cholesterol and plasma cytokine were determined.

Results: Consumption of the SFA diet resulted in increased expression of genes involved in inflammation processes in adipose tissue, without changes in morphology or insulin sensitivity. The MUFA diet led to a more anti-inflammatory gene expression profile, which was accompanied by a decrease in serum LDL-cholesterol concentrations and an increase in plasma and adipose tissue oleic acid content.

Conclusion: Consumption of an SFA diet resulted in a proinflammatory 'obese-linked' gene expression profile, whereas consumption of a MUFA diet caused a more anti-inflammatory profile. This suggests that replacement of dietary SFA by MUFA could prevent adipose tissue inflammation and may reduce the risk for inflammation-related diseases such as metabolic syndrome.

INTRODUCTION

The presence of the metabolic syndrome represents a risk factor for cardiovascular diseases and type 2 diabetes mellitus and is characterized by central obesity, insulin resistance, dyslipidemia and hypertension [1, 2]. The pathogenesis of the syndrome is not completely understood but involves a complex interaction between genetic and lifestyle factors [68, 69]. Diet is one of the lifestyle factors that has been changed in the past decades, and this might have contributed to the enormous increase in the prevalence of obesity. With this increasing prevalence of obesity, there is growing interest in adipose tissue biology.

In addition to its function as energy-storage organ, adipose tissue has important endocrine functions that are characterized by the release of adipocytokines and adipochemokines. Changes in adipose tissue mass are often accompanied by changes in production of these adipokines [70, 71]. Several studies have shown that obesity is accompanied by an increase in chronic low-grade inflammation in adipose tissue. This inflammation is characterized by macrophage infiltration and expression of inflammatory genes [72, 73]. Subsequent weight loss in obese subjects could reduce the gene expression of proinflammatory molecules, thereby improving the inflammatory status [50, 74]. The question is whether a change in diet without weight loss can result in similar changes in adipose tissue inflammatory status.

One of the dietary components that is known to be positively associated with concentrations of inflammation markers in plasma is saturated fatty acids (SFA) [75-77]. The replacement of an SFA-rich diet with a monounsaturated fatty acid (MUFA)-rich diet can cause a decrease in plasma inflammatory molecules [49, 78] and could thereby result in an improvement in insulin sensitivity and LDL-cholesterol concentrations [79, 80].

One of the ways through which fatty acids may exert their effects on inflammation is through activation of peroxisome proliferator-activated receptors (PPARs). PPARs have regulatory roles in lipid and glucose homeostasis and activation of PPAR can decrease inflammation [81]. Activation of PPAR by fatty acids increases with chain length and degree of unsaturation of the fatty acid [82]. It is plausible that a shift in type of dietary fat from SFA toward MUFA might decrease the expression of inflammatory genes in adipose tissue via PPAR.

To study the effects of changes in type of dietary fat on insulin sensitivity and adipose tissue functioning, we conducted a controlled-feeding trial in which we investigated the effect of an SFA-rich diet and an MUFA-rich diet on insulin sensitivity and whole-genome transcriptional changes in adipose tissue from healthy subjects at risk of metabolic syndrome.

SUBJECTS AND METHODS

Subjects

Our study population was a subgroup of the participants who were included in a previously reported controlled-feeding trial [83]. Twenty persons from this trial were selected on the basis of their willingness to participate in additional measurements. Inclusion criteria were: age between 45 and 60 years and body mass index (BMI; in kg/m^2) ≥ 25 or waist circumference ≥ 80 cm for women and ≥ 94 cm for men. Subjects were excluded if they were hypercholesterolemic subjects (total cholesterol ≥ 8mmol/L) or if they had non-treated diabetics according to World Health Organization criteria [84]. Subjects provided informed consent, and the Medical Ethical Committee of Wageningen University (Netherlands) approved the study.

Study design

All subjects consumed an SFA-rich run-in diet for two wk. After run-in, the subjects were allocated to one of the intervention diets, which they consumed for 8 wk. Ten subjects received an SFA diet, which was similar to the run-in diet, and 10 subjects received an MUFA-rich diet. The diets were comparable with regard to carbohydrates, protein, total fat and dietary fiber. The SFA diet contained 19% SFAs and 11% MUFAs and the MUFA diet contained 11% SFAs and 20% MUFAs, mainly in the form of refined olive oil. During the study, 90% of energy need was supplied by the diet on the basis of the energy requirements of the subjects. For the remaining 10%, subjects had a limited choice from low-fat products.
Subjects were advised to maintain their usual physical activity pattern. Body weight was monitored, and the level of energy intake was adjusted in order to prevent weight changes.

Body weight and body composition

Body weight, waist circumference and body composition were measured before and after intervention. Body composition was determined by air displacement plethysmography (BodPod; Life Measurement, Concord, CA)[85].

Assessment of insulin sensitivity

Insulin sensitivity was assessed by using the euglycemic-hyperinsulinemic clamp procedure as described by DeFronzo et al. [86]. The clamp measurements were performed before and during the run-in period and in the final 2 wk of the intervention. Glucose was clamped at a concentration of 5 mmol/L by titrating a variable infusion rate of glucose (20%) against a fixed infusion rate of insulin (Actrapid, Novo Nordisk

Farma BV, Alphen a/d Rijn, Netherlands, 40 $mU \cdot m^{-2} \cdot min^{-1}$). Glucose infusion rate was computed on the basis of glucose concentrations measured at 5-min intervals throughout the clamp. Once a steady state of 5 mmol/L glucose was reached for ≥ 30 min, whole-body insulin sensitivity (M-value) was determined and expressed in μmol $glucose \cdot kg\ fat\ free\ mass^{-1} \cdot min^{-1}$.

Adipose tissue

Subcutaneous adipose tissue samples were obtained by needle biopsy from the periumbilical area under local anesthesia. The samples were collected after an overnight fast, before the clamp measurement. The samples were rinsed to eliminate blood and two-thirds of the sample was immediately frozen in liquid nitrogen and stored at -80°C. The remainder was fixed in formalin, dehydrated and embedded in paraffin for histology.

Plasma fatty acid profile, cholesterol and plasma cytokines

Venous blood was collected after an overnight fast at the end of the run-in period and at the end of the intervention period. Plasma free fatty acids (FFAs) were measured by gas-liquid chromatography as previously described [87]. Plasma FFA fractions were expressed as a percentage of total FFA.

Concentrations of total and HDL-cholesterol were determined by a Dimension Clinical Chemistry System (Dade Behring Inc, USA). LDL-cholesterol was calculated by using the formula of Friedewald et al. [88].

Concentrations of adiponectin, complement 3 (C3) and Regulated upon Activation Normal T-cell Expressed and Secreted / Chemokine CC-motif Ligand 5 (RANTES/CCL5) were determined in plasma using the Luminex xMAP technology platform (Rules Based Medicine, Austin, Texas, USA).

Adipose tissue fatty acid composition.

Fatty acid composition of adipose tissue was measured according to the method of Deslypere et al [89]. Methylesters were prepared, separated by capillary gas chromatography, and detected with a flame ionization detector (Agilent 5890 Series II and 6890N).

Histologic analysis

Sections 3-μm thick were obtained from each adipose tissue sample. Hydrogen peroxide (3%) was added, followed by normal horse serum. The sections were incubated overnight (4°C) with anti-MAC-2 primary antibody (1:2000, Cedarlane Laboratories, Canada) and afterward for 30 min with a biotinylated horseradish

peroxidase-conjugated secondary antibody (horse anti-mouse IgG, Vector Laboratories, Burlingame, Canada). Histochemical reactions were performed by using Vector's Vectastain ABC Kit (Burlingame, CA) and Sigma Fast 3,3'-diaminobenzidine as substrate (Sigma, St. Louis, MO) [90]. Sections were counterstained with haematoxylin. For each subject we examined 3 adipose tissue sections before and 3 sections after intervention. Sections were of comparable size, and we counted the MAC-2-positive cells that were present in the sections.

RNA extraction

Total RNA was isolated from adipose tissue by using Trizol reagent (Invitrogen, Breda, NL) and purified by using the Qiagen RNeasy Micro kit (Qiagen, Venlo, NL). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, NL).

Microarray processing

Total RNA (500 ng/sample) was labeled by using a one-cycle cDNA labeling kit (MessageAmpTM II-Biotin Enhanced Kit, Ambion) and hybridized to human whole genome GeneChip arrays encoding 17.699 genes, designed by the European Nutrigenomics Organisation and manufactured by Affymetrix (Santa Clara, CA). Sample labeling, hybridization to chips, and image scanning was performed according to the manufacturers' instructions.

Microarray data analysis

Quality control was performed and fulfilled the criteria for array hybridization suggested by the Tumor Analysis Best Practices Working Group [91]. Microarrays were analyzed by using the reorganized oligonucleotide probes as described by Dai et al. [92]. All individual probes for a gene were combined, which allowed the possibility of detecting overall transcription activity, on the basis of the latest genome and transcriptome information, instead of on the basis of the Affymetrix probe set annotation. Expression values were calculated with the Robust Multichip Average (RMA) method and normalization was done by using quantile normalization [93, 94].

Only probe sets with normalized signals >20 on >5 arrays were defined as expressed and selected for analysis. Individual genes were defined as changed when comparison of the average normalized signal intensities showed a P-value <0.05 in a two-tailed paired t-test with Bayesian correction (Limma) [95].

Data were analyzed with the use of Ingenuity Pathway Analysis version 6.0 (Ingenuity Systems, Redwood City, CA), GenMAPP 2 (www.genmapp.org), Metacore version 4.7 (GeneGo Inc, Kanata, Canada) and Gene Set Enrichment Analysis

(http://www.broad.mit.edu/gsea/). Because the results were similar, only results from Ingenuity Pathway Analysis are displayed. Our analysis identified canonical pathways that were most significant to the data set. The analysis was performed for up- and down-regulated gene sets separately. Array data has been submitted to the Gene Expression Omnibus (accession no. GSE14954).

cDNA synthesis and quantitative real-time PCR

Quantitative real-time polymerase chain reaction (q-PCR) was used to confirm microarray data. First, 500 ng of total RNA was reverse transcribed with a Promega cDNA synthesis kit (Promega Benelux BV, Leiden, NL*).* cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen, Breda, NL) on a Biorad I-Cycler PCR machine. Primer sequences were chosen based on the sequences available in PRIMERBANK (http://pga.mgh.harvard.edu/primerbank/index.html). The mRNA expression of all genes was normalized to β-actin expression**.**

Statistical analysis

The statistical package SPSS (version 15.0; SPSS, Chicago, IL) was used for analysis of the data. Changes within diet groups were determined by paired t-tests. Differential changes between the diet groups were examined by unpaired t-tests.

RESULTS

Subjects

Baseline characteristics of the subjects are shown in table 1. No significant differences were observed, except for age, which was higher in the MUFA diet group (by 6.9 ± 2.6 y). All subjects completed the trial. Weight slightly decreased with the SFA diet (-1.3 ± 1.7 kg) and with the MUFA diet (-0.9 ± 1.3), but weight changes were not significantly different between the diet groups. Values are reported as means ±SDs.

Table 1 Baseline characteristics of the subjects

		SFA-rich diet (n=10)	MUFA-rich diet (n=10)
Age (y)		52.0 ± 6.3	58.9 ± 5.3*
Sex (M/F)		5 / 5	5 / 5
Body weight (kg)		79.8 ± 12.3	77.4 ± 14.2
BMI (kg/m$^{2)}$		26.1 ± 2.9	28.3 ± 6.6
Waist circumference (cm)	Men	102.0 ± 6.6	98.8 ± 6.5
	Women	89.0 ± 4.2	96.8 ± 23.3
Body fat percentage (%)	Men	28.0 ± 3.1	31.3 ± 5.9
	Women	36.6 ± 8.2	41.8 ± 12.1
HDL cholesterol (mmol/L)		1.25 ± 0.28	1.28 ± 0.26
Triglycerides (mmol/L)		1.06 ± 0.42	1.10 ± 0.34
Insulin sensitivity (μmol·kg FFM^{-1}·min^{-1})		52.47 ± 18.87	51.74 ± 12.48

All values are shown as means ± SDs

*significantly different from the SFA-rich diet group, P<0.05 (t-test)

Abbreviations: saturated fatty acid (SFA), monounsaturated fatty acid (MUFA)

Insulin sensitivity

Clamps were used to determine the effect of the diets on insulin sensitivity. Although average changes in insulin sensitivity within the diet groups were positive with both diets, no significant changes were observed within and between the diet groups (table 2).

Lipids

Mean (±SD) changes in plasma fatty acid composition are shown in table 2. Plasma total SFA (-4.04 ± 1.39%) was lower, whereas plasma total MUFA (6.23 ± 1.50%) and plasma oleic acid (6.15 ± 1.34%) were higher with the MUFA diet than with the SFA diet. No significant changes in total FFA concentrations were observed with the diets. The MUFA diet reduced serum total cholesterol (-0.60 ± 0.19 mmol/L) and LDL-cholesterol (-0.49 ± 0.13 mmol/L) compared with the SFA diet but did not affect serum HDL cholesterol (table 2).

Table 2 Changes in assessed variables due to the intervention

	Baseline[1]		Within group comparison[2]		Between group comparison (MUFA-SFA)	
	SFA diet (n = 10)	MUFA diet (n = 10)	SFA diet (n = 10)	MUFA diet (n = 10)	Difference[1]	P value
Insulin sensitivity ($\mu mol \cdot kg\ FFM^{-1} \cdot min^{-1}$)	52.47±18.87	51.74±12.48	5.40±10.14	7.79±16.32	2.38±6.08	0.70
Total cholesterol (mmol/L)	5.57±1.10	5.67±0.62	-0.04±0.23	-0.64±0.54*	-0.60±0.19	0.01
LDL-cholesterol (mmol/L)	3.94±0.94	3.99±0.63	-0.03±0.25	-0.52±0.34*	-0.49±0.13	<0.01
HDL-cholesterol (mmol/L)	1.25±0.28	1.28±0.26	-0.02±0.08	-0.10±0.17	-0.08±0.06	0.20
Plasma total FFA (g/L)	0.14±0.04	0.15±0.03	-0.01±0.04	-0.00±0.03	0.01±0.02	0.42
Plasma total SFA(%)	35.04±1.66	34.95±2.65	0.26±2.59	-3.79±3.54*	-4.04±1.39	0.01
Plasma total MUFA(%)	40.52±2.83	38.59±3.44	-2.15±4.10	4.07±2.37*	6.23±1.50	<0.01
Plasma oleic acid(%)	34.35±2.22	32.30±2.80	-1.97±3.70	4.18±2.09*	6.15±1.34	<0.01
Plasma total PUFA(%)	22.53±2.56	24.43±3.61	1.38±5.66	-0.08±3.90	-1.46±2.18	0.51
Adipose tissue total SFA(%)	30.00±2.52	29.92±2.17	0.09±0.73	-0.06±0.65	-0.15±0.31	0.63
Adipose tissue total MUFA(%)	51.32±2.90	51.45±2.23	-0.13±0.71	0.32±0.85	0.45±0.35	0.21
Adipose tissue oleic acid(%)	34.35±2.22	32.30±2.81	-0.20±0.65	0.50±0.57*	0.70±0.27	0.02
Adipose tissue total PUFA(%)	22.53±2.56	24.43±3.61	0.01±0.53	-0.08±0.30	-0.09±0.19	0.64
Adiponectin (µg/mL)	3.07±0.65	3.96±1.68	-0.20±0.35	-0.13±0.51	-0.07±0.19	0.72
Complement 3 (mg/mL)	0.90±0.12	0.96±0.11	-0.01±0.08	0.05±0.13	-0.07±0.05	0.20
RANTES/CCL5 (ng/mL)	5.49±4.66	8.51±5.43	-4.16±5.08	-7.08±5.65	2.95±2.41	0.24

[1]Values are means ± SDs, [2]Values are mean changes ± SDs, *changed within the diet group ($p<0.05$), within group comparison were made by paired t test; between-group comparisons were made by standard t test. Abbreviations: Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA), Polyunsaturated fatty acids (PUFA), free fatty acid (FFA), Free fatty acid (FFM), Regulated on activation normal T cell expressed and secreted/chemokine (C-C motif) ligand 5 (RANTES/CCL5)

Adipose tissue fatty acid composition

Fatty acid profiling in adipose tissue was used to determine incorporation of dietary fatty acid composition in adipose tissue (table 2). Similar to plasma fatty acid composition, the oleic acid fraction of the adipose tissue fatty acids was significantly higher (0.70 ± 0.27%) upon the MUFA diet than with the SFA diet.

Microarray analysis

Microarray analysis was performed on adipose tissue before and after intervention. From the 16314 genes on the array, 13280 genes were expressed in adipose tissue (figure 1). Intervention with the SFA diet resulted in changed expression of 1523 genes whereas intervention with the MUFA diet resulted in changed expression of 592 genes. Of these genes, 76 were differentially expressed on both diets. Similar numbers of genes were up- and down-regulated. To gain further insight into the role of these genes, pathway analysis was performed. A total of 38 canonical pathways were altered by the SFA diet whereas 12 pathways were regulated by the MUFA diet (figure 1). The top

ten most significantly up and downregulated pathways for each diet are listed in figure 2. For the MUFA diet, only 5 pathways were significantly up-regulated. Consumption of the SFA diet resulted in the most strongly regulated pathways, which were mainly up-regulated and primarily included processes related to immune function and inflammation. The down-regulated pathways included amino acid metabolism and fatty acid metabolism. The genes that were down-regulated within the pathway of fatty acid metabolism are mainly involved in fatty acid oxidation and triglyceride synthesis.
Consumption of the MUFA diet resulted in an up-regulation of phospholipid metabolism and degradation and eicosanoid signaling and in a down-regulation of the complement system and pyruvate metabolism. Sterol regulatory element-binding protein-1 (SREBP-1) and SREBP-1-dependent genes involved in lipogenesis were down-regulated as well.

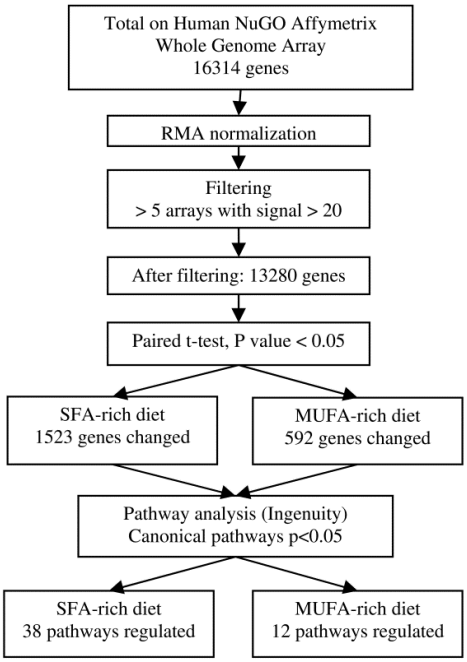

Figure 1 Selection of genes in microarray analysis. Abbreviations: Robust Multichip Average (RMA), Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA)

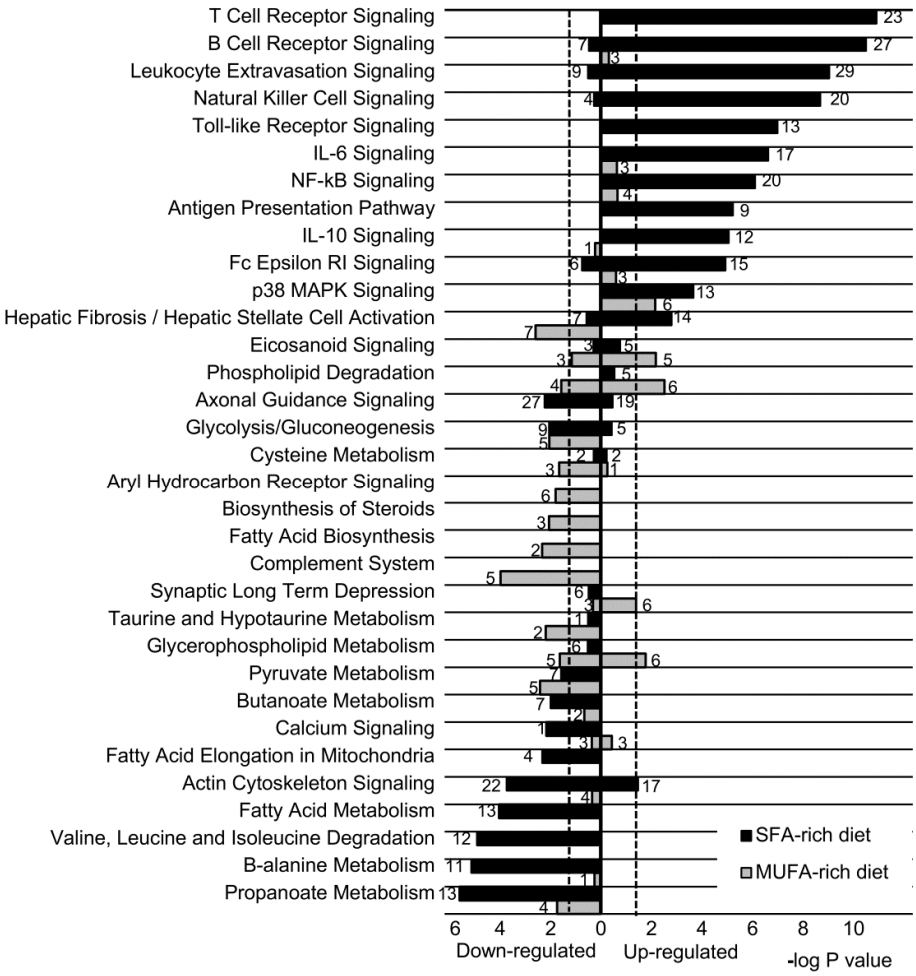

Figure 2 Top 10 significantly up- and down-regulated canonical pathways of each diet according to Ingenuity Pathway Analysis. Black bars indicate the saturated (SFA)-rich diet, and grey bars indicate the monounsaturated (MUFA)-rich diet. Numbers next to the bars indicate the number of significantly changed genes involved in each pathway. The significance of each pathway is indicated by –log(P value) >1.3 which corresponds to P<0.05. The dashed vertical line crosses the x-axis at –log(P value) = 1.3

The individual expression changes of genes involved in the most markedly altered immune pathways are depicted in a heatmap in supplemental data figure 1 (http://www.ajcn.org/content/90/6/1656/suppl/DC1). Overall, consumption of an SFA diet for 8 wk resulted in an increased expression of genes involved in immune

processes whereas the same genes were not changed or down-regulated after consumption of the MUFA diet.

Several genes that are known to be involved in inflammation and that were not present in the pathways in figure 2 were also up-regulated with an SFA diet and are shown in figure 3. These included markers for recruitment of leukocytes, genes involved in prostaglandin synthesis and monocyte/macrophage markers. Consumption of the MUFA diet, however, resulted in a down-regulation of several adipose tissue macrophage genes.

In concordance with the increase in expression in markers of inflammation, expression of the antiinflammatory adipokine adiponectin (ADIPOQ) and transcription factor PPARγ was decreased on the SFA diet. Clear differences in individual responses to the diets are also shown in figure 3.

Gene	Gene Description	ID
CCR1	chemokine (C-C motif) receptor 1	1236
CXCL10	chemokine (C-X-C motif) ligand 10	3627
CCL5	chemokine (C-C motif) ligand 5	1230
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	3117
CCL4	chemokine (C-C motif) ligand 4	6352
CCR7	chemokine (C-C motif) receptor 7	9332
ADAM8	ADAM metallopeptidase domain 8	101
EMR1	egf-like module containing, mucin-like, hormone receptor-like 1	2015
CTSS	cathepsin S	1520
IL6R	interleukin 6 receptor	3570
PTGS1	prostaglandin-endoperoxide synthase 1	5742
CD163	CD163 molecule	4345
IL10RA	interleukin 10 receptor, alpha	3587
CD209	CD209 molecule	30835
HLA-DQB2	major histocompatibility complex, class II, DQ beta 2	3120
CD86	CD86 molecule	942
CSF1R	colony stimulating factor 1 receptor	1436
IL4R	interleukin 4 receptor	3566
HLA-A	major histocompatibility complex, class I, A	3105
HLA-DMA	major histocompatibility complex, class II, DM alpha	3108
HLA-DMB	major histocompatibility complex, class II, DM beta	3109
IL18	interleukin 18	3606
ADIPOQ	adiponectin	6351
PPARg	peroxisome proliferator-activated receptor gamma	5468
CXCR4	chemokine (C-X-C motif) receptor 4	7852
IL7R	interleukin 7 receptor	3575
MCP1R	monocyte chemoattractant protein 1 receptor	729230
IL1R2	interleukin 1 receptor, type II	7850
IL8RA	interleukin 8 receptor, alpha	3577
IL8	interleukin 8	3576
PTGS2	prostaglandin-endoperoxide synthase 2	5743
IL8RB	interleukin 8 receptor, beta	3579

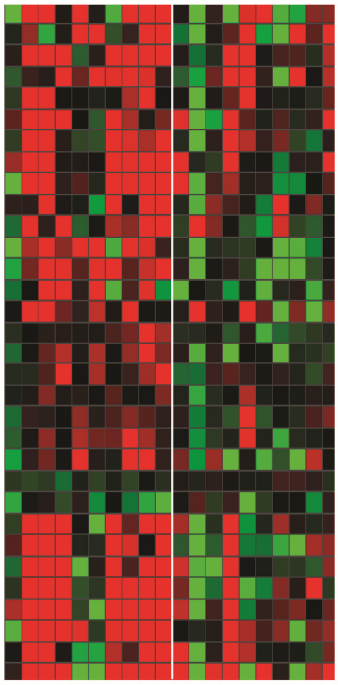

Figure 3 Gene expression changes of other well-known immune related genes, which were significantly changed with one of the diets (paired t-test with Bayesian correction). Each row represents a single gene, each column represents a single person in a diet group. The expression scale is $\log_2$-based and ranges from ≤-0.5 (green) to ≥ 0.5 (red).

Immunohistochemistry

To investigate whether the diet-induced gene expression changes also resulted in protein or morphological changes in adipose tissue, immunohistochemical analysis for MAC-2 was performed. The adipose tissue seemed apparently healthy, and only a few MAC-2-positive cells that formed characteristic crownlike structures was seen for only one person who received the SFA diet (supplemental data figure 2 http://www.ajcn.org/content/90/6/1656/suppl/DC1).

Confirmation of gene expression data

To confirm gene expression changes revealed by microarray analysis, q-PCR was used. Genes for q-PCR were selected based on their role in different inflammatory processes. Expression of ADIPOQ, RANTES/CCL5, CD14, CD163 and ras-related C3 botulinum toxin substrate 2 (RAC2) also significantly changed with q-PCR (figure 4). The change in expression of complement subcomponent C1q (C1QB), cathepsin S (CTSS) ,integrin beta 2 (ITGB2) and PPARγ showed the same direction and extent of change as the microarray data but was not significant.

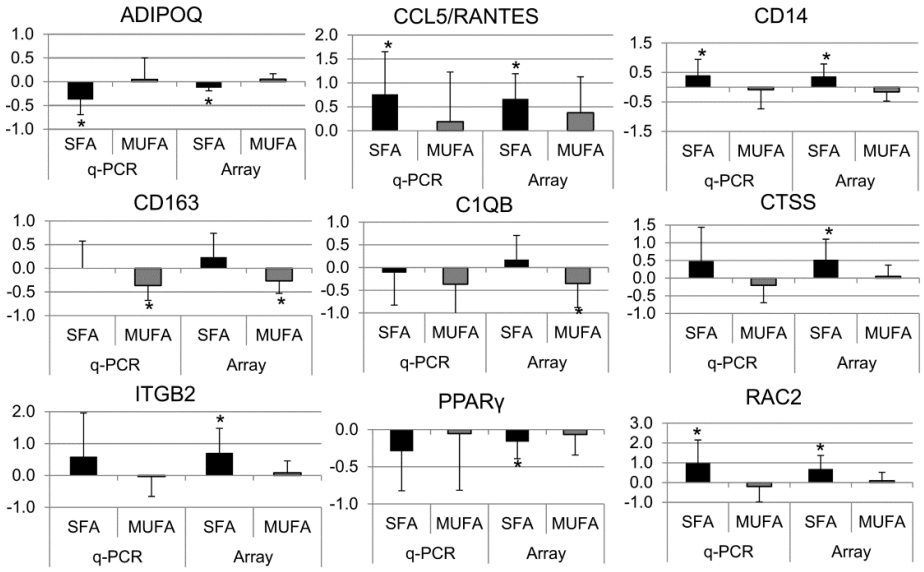

Figure 4 Validation of genes with quantitative real-time polymerase chain reaction (q-PCR). Black bars represent the mean (±SD) log_2-transformed expression changes with the saturated fatty acid (SFA)-rich diet after intervention and grey bars represent the log_2-transformed expression changes with the monounsaturated fatty acid (MUFA)-rich diet. For q-PCR data, significance was determined by using a paired t-test. *significantly different, $P<0.05$.

Adiponectin, Complement 3 and RANTES/CCL5 in plasma

To elucidate whether changes observed in adipose tissue for gene expression level were also reflected by changes in plasma, concentrations of adiponectin, C3 and RANTES/CCL5 were measured. In concordance with the gene expression in adipose tissue, plasma adiponectin concentrations decreased in 8 out of 10 subjects with the SFA diet (-0.20 ± 0.35 μg/mL) and did not change with the MUFA diet (-0.13 ± 0.51 μg/mL). Plasma C3 concentrations were not changed (-0.01 ± 0.08 and 0.05 ± 0.13 mg/mL for the SFA and MUFA diets, respectively) and plasma RANTES/CCL5 concentrations were significantly decreased with both diets (-4.16 ± 5.08 and -7.08 ± 5.65 ng/mL for the SFA and MUFA diets, respectively).

DISCUSSION

Several studies have shown increased inflammation in adipose tissue of obese subjects and revealed its possible role in the development of insulin resistance [96-98]. Our study suggests that the type of dietary fat is an important component in the cause of adipose tissue inflammation. We showed in a controlled-feeding trial that consumption of an SFA diet results in increased expression of genes involved in inflammatory processes, whereas consumption of an MUFA diet results in a decreased expression of inflammatory genes in adipose tissue of abdominally overweight subjects. Consumption of the diets did not change insulin sensitivity, but total and LDL cholesterol concentrations were decreased with the MUFA diet.

Some of the inflammation processes that were strongly up-regulated on the SFA diet were T and B cell receptor signaling and leukocyte extravasation signaling. This could be indicative of increased attraction of immune cells toward the adipose tissue with subsequent infiltration of the tissue and increased local inflammation. It is well known that macrophages can infiltrate adipose tissue in obese subjects, and the presence of T-cells in adipose tissue of obese and diabetic mice and humans has recently been confirmed [99-101]. In addition to the up-regulation of lymphocyte-related processes, up-regulation of macrophage markers was observed with the SFA diet. Consumption of the MUFA diet resulted in antiinflammatory effects because the expression of genes involved in complement system and expression of macrophage marker genes were decreased. For both diets immunohistochemical staining of macrophages showed no changes in macrophage infiltration in the adipose tissue samples. In addition to inducing expression of genes involved in inflammation, the SFA diet reduced expression of genes involved in fatty acid β-oxidation and triglyceride synthesis. This could point to decreased lipid handling capacity of the adipocytes, possibly as a cause or a consequence of increased inflammation.

So far, inflammation in adipose tissue is reported to be present predominantly in obesity. Microarray expression profiling of adipose tissue from obese and non-obese Pima Indians showed an over-expression of genes involved in immune processes in obese subjects [52, 73]. Many of these overexpressed genes, such as CTSS, IL-8 and ITGB2, were also up-regulated in our study in response to the SFA diet. In addition, adiponectin expression in adipose tissue and adiponectin plasma concentration, which are usually decreased in obesity, were lowered on the SFA diet [102]. Furthermore, weight loss in obese subjects resulted in decreased expression of genes involved in T and B cell receptor signaling [100], whereas we observed that consumption of the SFA diet induced these processes [74, 100].

In consideration of the similarities between the gene expression profiles in obesity and gene expression changes due to the SFA diet, we hypothesize that consumption of the SFA diet leads to an obese-linked gene expression profile in the adipose tissue without obtaining an obese phenotype. Our study subjects were non-diabetic, were only moderately overweight, and did not gain weight during the study, indicating that the observed up-regulation of inflammatory processes in their adipose tissue was not due to weight gain but can only be explained by consumption of the SFA diet. A high SFA intake is associated with elevated concentrations of plasma inflammation markers [75-77] but has never been shown to induce inflammation-related gene expression in adipose tissue.

We observed a large interindividual variation in response to the diets. Possible reasons for this could be the genetic and epigenetic variation between the subjects. [103]. In addition, adipose tissue samples were obtained before completing the run-in period, and dietary patterns before the study might have influenced the individual response. On the basis of the outcome of our study, we speculate that subjects were consuming less SFA before start of the study than was provided by our intervention.

On the basis of former studies [17, 79, 80], we hypothesized that consumption of an MUFA diet would improve insulin sensitivity and cholesterol concentrations accompanied by gene expression changes in processes contributing to improvements in health. The MUFA diet did not affect insulin sensitivity but reduced total and LDL cholesterol. In addition, a change to a more unsaturated fatty acid profile in both plasma and adipose tissue was observed, accompanied by the down-regulation of proinflammatory genes in adipose tissue. It is hard to distinguish within this study whether the beneficial effects observed with the MUFA diet are due to increased MUFA content or decreased SFA content; it could be a combination of both.

Despite increases in inflammatory gene expression, which is associated with decreased insulin sensitivity [104], we did not observe changes in insulin sensitivity. A possible reason might be that the intervention period was too short to induce significant changes. Yet, this also shows the high potential for use of gene expression profiles as a sensitive and early marker for nutrition-induced changes.

The changes in adiponectin plasma concentration are in line with the gene expression changes in adipose tissue, whereas the changes in plasma RANTES/CCL5 and C3 are not. In contrast to adiponectin, RANTES/CCL5 and C3 are not secreted only by adipose tissue but also by other tissues that are apparently not affected in this early inflammatory stage. Furthermore, an increase in inflammatory gene expression in adipose tissue does not necessarily result in adverse effects on whole body level. A controlled immune response indicates that the body is able to react properly to certain triggers, thereby protecting itself from infections and diseases. We hypothesize that the

observed gene expression changes while on the SFA diet are early local responses restricted to the adipose tissue. Prolonged consumption of an SFA diet might result in more systemic effects leading to adverse health effects on whole-body level.

The question remains how consumption of an SFA diet could lead to an 'obese' proinflammatory gene expression profile in adipose tissue of healthy, abdominally overweight subjects. In obesity, inflammation is usually accompanied by increased plasma fatty acids and increased adipose tissue mass. Both can result in increased inflammation, either due to hypoxia in the expanding adipose tissue, or due to lipid overload in various organs [98, 104]. However, we did not observe changes in total plasma FFA concentrations or changes in total adipose tissue mass for either of the diets; nor did we observe changes in expression of genes involved in hypoxia. A possible mechanism for the changed expression of inflammatory genes may be that the consumed fatty acids have different affinities to bind and activate immune processes regulating receptors such as the Toll like receptors (TLR) and nuclear receptors such as PPARs. It is known that SFA can modulate TLR activation and the expression of target genes of these receptors, which are involved in the inflammatory response [105, 106]. In our study, we observed that the SFA diet induced expression of different TLRs, the adaptor protein myeloid differentiation primary response gene 88 *(MYD88)* and its target genes, e.g. *IL-8, RANTES/CCL5*, chemokine ligand 4 (*CCL4*), *CD86*, prostaglandin-endoperoxide synthase 2 (*PTGS2*) and chemokine ligand 10 (IP-10). This could point toward a role of the TLR pathway in SFA-mediated gene expression. Another finding is the down-regulation of the transcription factor *PPARγ* and several target genes- ie lipoprotein lipase (*LPL*), 1-acylglycerol-3-phosphate O-acyltransferase 2 (*AGPAT2*) and adiponectin. SFA are poor ligands for PPARγ [107], and because PPARγ activation results in the down-regulation of inflammation, a decreased activation of PPARγ by SFA might lead to increased inflammation. This could suggest a possible role of PPARγ in the SFA-mediated expression of genes involved in inflammation.

In conclusion, our study shows that consumption of an SFA diet, compared with a MUFA-rich diet, leads to a proinflammatory obese-linked gene expression profile in adipose tissue of persons at risk for metabolic syndrome. Adipose tissue gene expression appears to be affected by diet before adipose tissue morphology or insulin sensitivity are changed and might be one of the first hallmarks in the development of metabolic syndrome.

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CHAPTER 3

The effects of a diet high in monounsaturated fat and a Mediterranean diet on PBMC whole genome gene expression and plasma proteins

SJ van Dijk, EJM Feskens, MB Bos, LCPGM de Groot, JHM de Vries,
M Müller and LA Afman

ABSTRACT

Background: The Mediterranean diet is considered as health promoting, the effect often being ascribed to its high content of monounsaturated fatty acids (MUFA) and polyphenols. These bioactive compounds can affect gene expression and might in this way regulate pathways and proteins related to cardiovascular disease prevention.
Objective: This study aimed to identify the effects of replacement of saturated fat (SFA) by MUFA in a western-type diet and the effects of a full Mediterranean (MED) diet on whole genome peripheral blood mononuclear cell (PBMC) gene expression and plasma protein profiles.
Design: Abdominally overweight subjects were allocated to a 8 wk completely controlled SFA-rich diet, a SFA-by-MUFA-replaced diet (MUFA diet) or a MED diet. Concentrations of 124 plasma proteins and PBMCs whole genome transcriptional profiles were assessed.
Results: Consumption of the MUFA and MED diet, compared with the SFA diet, decreased expression of oxidative phosphorylation (OXPHOS) genes, serum lipids and plasma Connective Tissue Growth Factor, myoglobin and Apo B concentrations. The MED diet additionally lowered plasma α-2-macroglobulin concentration compared with the SFA diet. Within the MED diet group concentrations of several pro-inflammatory proteins were lowered.
Conclusion: We conclude that MUFA as replacement of SFA in a western-type diet had similar effects on lowering expression of OXPHOS genes as in a MED diet. We hypothesize that replacement of SFA by MUFA increased metabolic health as reflected by lowered serum lipids and certain plasma proteins, thereby reducing metabolic stress and OXPHOS activity in PBMCs. The MED diet may have additional anti-atherogenic effects by lowering concentrations of pro-inflammatory plasma proteins.

INTRODUCTION

The importance of a healthy and balanced diet in the prevention of chronic diseases such as diabetes, cardiovascular disease (CVD) and cancer has been extensively studied and several dietary components with positive or negative health effects have been identified. Most studies have focused on the relation of single dietary components with disease, whereas a diet is a mixture of many dietary components that can interact with each other [108]. Nutritional scientists and institutions stimulate the translation from nutrient-based recommendations to food based recommendations [109] [110]. The Mediterranean diet has received considerable attention in this respect. It is characterized by a high intake of olive oil, fruits, vegetables, legumes, nuts, unrefined cereals, moderate intake of wine, and low to moderate intake of dairy products, meat, poultry, and fish [8, 111]. In spite of the relatively high fat content in the form of olive oil, consumption of the diet has been associated with a lower risk for CVD and metabolic syndrome [10, 112]. Furthermore, it has been associated with lower risk for certain types of cancer and with positive effects on cognitive function and longevity [7, 111-114]. Consumption of a Mediterranean diet has shown to affect CVD risk by lowering serum LDL cholesterol, increasing serum HDL cholesterol, improving blood pressure, hemostasis and endothelial function, but it is also suggested to influence inflammation and oxidative stress [17, 49, 115-118]. Virgin olive oil was identified as one of the main health promoting components of the Mediterranean diet, due to its high content of monounsaturated fat (MUFA) and polyphenols [118, 119].

The underlying mechanism of the positive effects of olive oil and the Mediterranean diet are still not fully understood. Components of the Mediterranean diet may execute their positive effects by decreasing circulating inflammatory and vascular markers in plasma and by decreasing expression of activation markers on PBMCs [49, 120-122]. In part this can be done by activation of cellular adaptive response pathways as numerous components of the diet are ligands of transcription factors that control gene expression of large sets of target genes [123]. We recently showed that longer term intake of distinct types of fatty acids had differentiating effects on gene expression profiles in PBMCs [59] and others showed that virgin olive oil consumption can alter PBMC gene expression [124, 125]. As PBMCs are of pivotal importance in the development of atherosclerosis characterization of the effects of consumption of the Mediterranean diet and of the MUFA component of the diet on whole genome gene expression changes in PBMCs may reveal leads towards possible molecular mechanisms.

We investigated the effects of replacement of SFA by MUFA from refined olive oil in a western type diet and the effects of a full MED diet high in MUFA from extra virgin

olive oil on whole genome PBMC gene expression. We also measured the effects of these diets on 124 plasma proteins implicated in chronic diseases, inflammation, endothelial function and metabolism. Our study will enable the differentiation between the effects of a single food component such as MUFA from olive oil and the effects of a mixture of foods such as in the MED diet on both PBMC gene expression and plasma proteins.

SUBJECTS AND METHODS

Subjects

In this study 60 healthy men and women were initially included. Inclusion criteria were age between 45 and 60 years and body mass index ≥ 25 kg/m^2 or waist circumference ≥ 80 cm for women and ≥ 94 cm for men. Subjects were excluded if they were hypercholesterolemic (total cholesterol ≥ 8 mmol/L), used anti-hypertensive or cholesterol-lowering medication or if they had non-treated diabetes according to World Health Organization criteria [84]. Subjects provided informed consent, and the Medical Ethical Committee of Wageningen University (the Netherlands) approved the study.

Study design

All subjects consumed a western type diet high in SFA (19 en %) for a two weeks run-in period to standardize dietary conditions. After run-in, the subjects were allocated to one of the intervention diets, which they consumed for 8 weeks. Subjects received either the western type SFA diet, which was similar to the run-in diet, a western type diet in which SFA was largely replaced by MUFA, mainly from refined olive oil (MUFA diet), or a Mediterranean type diet (MED diet) high in MUFA from extra virgin olive oil and other Mediterranean components (i.e. fatty fish, unrefined grain products, nuts, legumes and red wine). During the study, 90% of the energy need of the subjects was supplied by the test diet, for the remaining 10% subjects had limited choice of low-fat products which they all recorded. The composition of the diets is shown in table 1. Values are based on chemical analysis of complete duplicate diets plus the calculated contribution of the recorded low-fat products. The duplicate diets were collected daily for one energy level in each intervention group and pooled over all days. Subjects were advised to maintain their usual physical activity pattern. Body weight was monitored, and energy intake was adjusted to prevent weight changes.
A more detailed description of the study design and the diets has been published previously [117].

Blood collection and PBMC isolation

Fasting venous blood samples were collected at baseline, which is at the end of the run-in period, and after 8 weeks diet intervention. Immediately after blood collection, PBMCs were isolated by using the BD Vacutainer Cell Preparation Tubes according to the manufacturer's instructions.

Table 1 Mean daily intake of energy and nutrients of subjects on the SFA, MUFA, or MED diets according to duplicate portions plus recorded foods.

	SFA diet	MUFA diet	MED diet
Energy (MJ/d)	10.7	10.4	10.3
Total fat (energy-%)	36.8	39.9	40.2
SFA (energy-%)	19.2	10.9	10.7
MUFA (energy-%)	10.7	20.3	21.4
PUFA (energy-%)	5.4	7.0	6.5
n-3 PUFA (energy-%)	0.4	0.6	0.8
n-6 PUFA (energy-%)	5.0	6.4	5.6
Protein (energy-%)	13.5	11.4	14.5
Carbohydrates (energy-%)	47.3	46.4	41.1
Dietary fibre (g/MJ)	2.4	2.4	3.6
Cholesterol (mg/MJ)	25.7	24.2	25.6
Alcohol (energy-%)*	2.3	2.2	4.2

*Red wine was provided in the MED diet group. Alcohol consumption (maximum 2 glasses/day) was allowed in the other diet groups as well, with the exception of red wine.
Abbreviations: Saturated Fatty Acids (SFA), Monounsaturated Fatty Acids (MUFA), Mediterranean (MED) and Polyunsaturated Fatty Acids (PUFA).

RNA isolation and microarray analysis

RNA was isolated of all PBMC samples by using the Qiagen RNeasy Micro kit (Qiagen, Venlo, Netherlands). The RNA yield was quantified with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and RNA integrity was checked on an Agilent 2100 bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom). Samples were only accepted for microarray analyzes if the RNA integrity number (RIN) was > 8.

Total RNA (500 ng/sample) was labeled by using an one-cycle cDNA labeling kit (MessageAmpTM II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk a/d IJssel, Netherlands) and hybridized to human whole-genome GeneChip arrays encoding 17,699 genes, designed by the European Nutrigenomics Organization (NuGO) and manufactured by Affymetrix (Santa Clara, CA). Sample labeling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

Microarray data analysis

Results of quality control showed that 98 microarrays fulfilled our criteria. Microarrays were analyzed by using the reorganized oligonucleotide probes as described by Dai et al (29). All individual probes for a gene were combined, which allowed the possibility of detecting overall transcription activity on the basis of the latest genome and transcriptome information instead of on the basis of the Affymetrix probe set

annotation. Expression values were calculated with the Robust Multichip Average (RMA) method and normalized by using quantile normalization (30, 31). Only probe sets with normalized signals >20 on >5 arrays were defined as expressed and selected for analysis. Genes were defined as differently changed between the diets when the average normalized signal intensities showed a P value <0.05 in one-way ANOVA. Data were analyzed with the use of Ingenuity Pathway Analysis version 8.5 (Ingenuity Systems, Redwood City, CA) to identify canonical pathways that were most significant to the data set. Array data have been submitted to the Gene Expression Omnibus (GSE30509).

Serum lipids

Concentrations of triglycerides, total and HDL cholesterol were determined by a Dimension Clinical Chemistry System (Dade Behring Inc, Newark, NJ). LDL cholesterol was calculated by using the formula of Friedewald et al [88].

Plasma proteins

Concentrations of a set of 124 proteins were measured in fasting plasma before and after the intervention by multiplex immunoassay (Rules Based Medicine Inc, Austin, Texas, USA). The set of proteins present on this assay consists of proteins that are implicated in chronic diseases, inflammation, endothelial function and metabolism. In online supplemental table 1 all measured proteins are listed (http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0014422#s) Before analysis, 16 out of 124 proteins were removed from the dataset as the concentrations of these proteins were below the detection limit in more than half of the samples. For the remaining 108 proteins, values below the detection limit were replaced by 0.1*Least Detectable Dose. As IL-6 was one of the removed proteins and IL-6 was reported to be lowered after consumption of Mediterranean type of diets [49, 120], this protein was separately measured by high-sensitive enzyme immunoassay (Human IL-6 Quantikine HS ELISA Kit, R&D Systems, Abingdon, United Kingdom). Apo lipoprotein B (ApoB) was not included in the Human Multi-Analyte Profiles, and ApoB levels were additionally measured on a Hitachi 912 autoanalyser (Roche, Lelystad, The Netherlands) using a commercially available kit (Roche cat. nr.1551779). In total, 110 proteins were included in the analysis.

Statistical analysis

The statistical package PASW (version 17.0; SPSS Inc, Chicago, IL) was used for analysis of the data. Differential changes between the diet groups were examined using one-way ANOVA followed by a Least Significance Difference (LSD) post hoc test.

Changes within diet groups were determined by paired t-tests. For not normally distributed data changes between groups were examined using the Kruskal-Wallis test and changes within subject groups were examined using the Wilcoxon signed-rank test. LSD post hoc tests for non-normally distributed variables were performed on rank scores for these variables.

RESULTS

From the 60 subjects entering the study, 57 subjects completed the study. For 49 of these 57 subjects protein concentrations were measured and high-quality microarray analyses were performed on PBMCs collected before and after the intervention (supplemental figure 1). Baseline characteristics of these 49 subjects are shown in table 2. Adherence to the intervention diets caused small changes in body weight, but these changes were not significantly different between diet groups. Serum LDL cholesterol was reduced both by the MUFA diet (-0.40 mmol/L, 95%CI: -0.69,-0.12) and the MED diet (-0.54 mmol/L, 95%CI:-0.83, -0.25) compared with the SFA diet. The MED diet additionally increased HDL cholesterol (0.12 mmol/L, 95%CI 0.02, 0.21) compared with the MUFA diet. Triglyceride levels were reduced by the MED diet compared with the SFA diet (-0.32 mmol/L, 95% CI:-0.48; -0.15) and the MUFA diet (-0.24 mmol/L, 95%CI: -0.41; -0.07). An extensive analysis of triglcyerides, cholesterol and other metabolic parameters measured in all 57 subjects has been published previously [117].

Table 2 Baseline subject characteristics

	SFA diet (N=17)	MUFA diet (N=17)	MED diet (N=15)
Sex (m/f)	8 / 9	8 / 9	6 / 9
Age (years)	52.2 ± 6.9	58.4 ± 5.3	55.6 ± 6.5
BMI (kg/m^2)	26.3 ± 2.8	27.8 ± 5.6	29.5 ± 6.4
Total cholesterol (mmol/L)	5.66 ±1.14	5.86 ± 0.67	5.75 ± 1.07
LDL cholesterol (mmol/L)	4.00 ± 0.99	4.01 ± 0.65	3.99 ± 0.96
HDL cholesterol (mmol/L)	1.27 ± 0.27	1.37 ± 0.35	1.21 ± 0.39
Triglycerides (mmol/L)	1.08 ± 0.51	1.27 ± 0.49	1.52 ± 0.78

Data is displayed as mean ± standard deviation

Abbreviations: Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA), Mediterranean (MED)

PBMC microarray analyzes

A total of 656 genes was differently expressed between the three diets (ANOVA p-value<0.05). Post-hoc tests revealed that 372 genes were differently regulated between the MUFA and SFA diet and 216 genes were differentially regulated between the MED and the SFA diet.

Ingenuity pathway analysis identified canonical pathways that were significant to our dataset and figure 1 shows the ten most significant pathways. The three most differentially regulated pathways between the diets were oxidative phosphorylation (OXPHOS), mitochondrial dysfunction and ubiquinone biosynthesis. To visualize the effects of each diet, average expression changes per diet of the genes presented in these pathways are shown in figure 2. This figure illustrates that the MUFA diet mainly caused a down regulation of genes involved in mitochondrial OXPHOS and most of these genes were also down regulated with the MED diet, although to a lower extent. Most of the other differentially regulated pathways were signaling pathways. Diet effects on these pathways were less univocal.

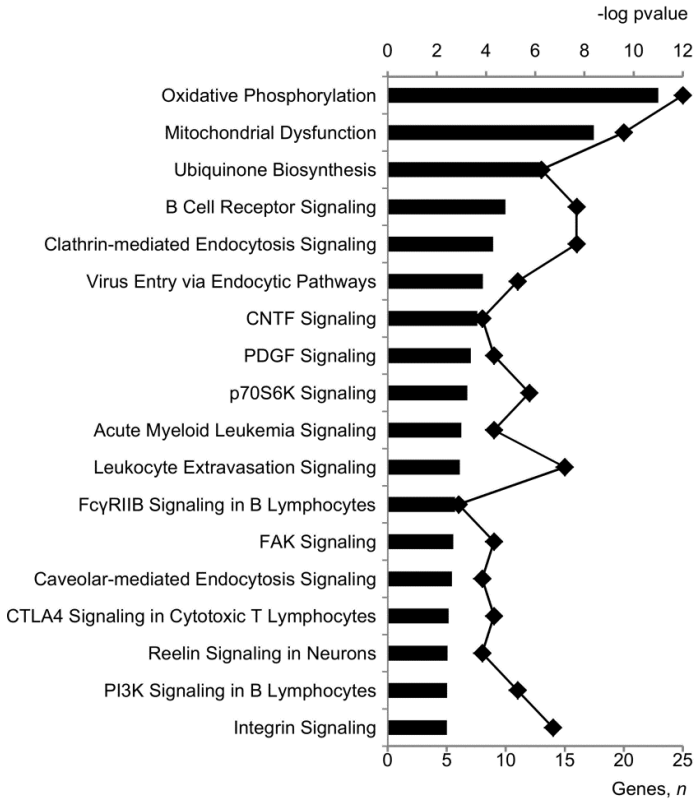

Figure 1 Ten most significantly changed pathways between the SFA, MUFA and MED diets as analyzed by Ingenuity Pathway analysis. Horizontal bars indicate the –log (p value), squares indicate the number of differently regulated genes within a pathway.

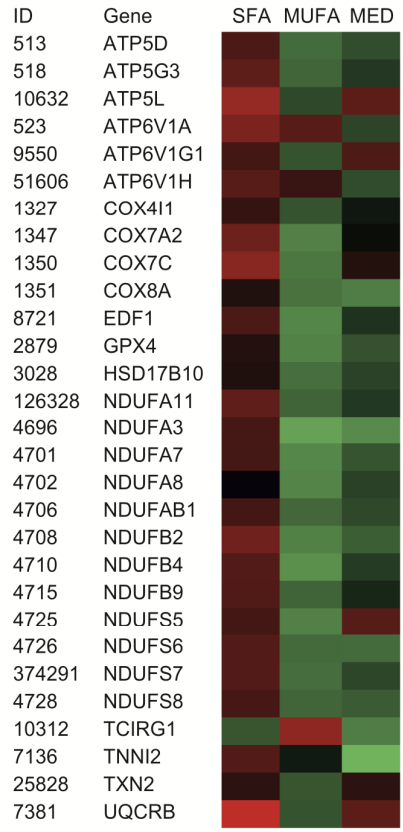

Figure 2 Average expression changes of genes in the most significant differently regulated pathways (oxidative phosphorylation, mitochondrial dysfunction, ubiquinone biosynthesis). Average changes in expression (signal log ratios) for each diet group are displayed on a color scale, ranging from ≤ -0.25 (green) ≥ 0.25 (red).

Plasma proteins

Of the 110 plasma proteins analyzed, seven proteins were differently changed by the diets (table 3) and included Alpha-2-Macroglobulin (A2M), Apolipoprotein B (ApoB), Connective Tissue Growth Factor (CTGF), Interleukin 12p70, Myoglobin, Sex Hormone Binding Globulin (SHBG) and Thyroxine Binding Globulin (TBG). Posthoc analyzes showed that compared with the SFA diet both the MUFA and MED diet reduced concentrations of ApoB, CTGF and myoglobin. Additional effects of the MUFA diet were observed for TBG and SHBG concentrations that increased and for IL12p70 concentration that decreased. An additional effect of the MED diet was found for A2M which concentration decreased compared with the SFA diet. TBG

concentrations in the MUFA diet were not only increased compared to the SFA but also to the MED diet. Concentrations of all proteins before and after intervention are displayed in supplemental table 2. Concentrations of several pro-atherogenic and pro-inflammatory proteins such as IL1β, Factor VII, macrophage inflammatory protein (MIP1α), Serum Amyloid P (SAP), Thrombopoietin (TPO) and Vascular Endothelial Growth Factor (VEGF) were significantly lowered in subjects within the MED diet group, but these changes were not significantly different from the smaller changes observed in the other groups.

Table 3 protein concentrations of differentially changed proteins before and after diet intervention

	SFA diet		MUFA diet		MED diet		Between diets
Protein	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention	P value [#]
A2M (mg/mL)	0.83±0.17	0.85±0.16	0.81±0.18	0.78±0.15	0.85±0.21	0.75±0.14*	<0.05[b]
Apo B (mg/mL)	1.09±0.27	1.12±0.29	1.10±0.17	1.03±0.20*	1.15±0.24	1.02±0.24*	<0.005[a,b]
CTGF (ng/mL)	2.02±0.72	2.11±0.94	2.6±1.01	2.23±0.88*	2.09±1.24	1.75±1.11*	<0.05[a,b]
IL12p70 (pg/mL)	70.7±38.9	77.8±37.0	70.3±20.2	66.1±21.1	65.8±15.6	65.5±13.1	<0.05[a]
Myoglobin (ng/mL)	10.0 (8.4,14.5)	12.4 (9.4,15.0)	11.8 (8.9,16.2)	10.8 (7.9,15.4)[†]	12.3 (9.4,16.0)	11.6 (9.0,13.2)	<0.05[a,b]
SHBG (nmol/L)	51.7±28.5	48.0±23.9*	39.8±15.9	41.4±18.9	36.5±15.0	36.5±14.9	<0.05[a]
TBG (µg/mL)	52.8±14.9	51.7±12.3	54.6±9	57.8±10.5*	57.5±16.6	54.8±15.0*	<0.05[a,c]

Data is displayed as mean ± standard deviation

* significantly different from baseline (p<0.05 in paired t-test)

[†] significantly different from baseline (p <0.05 in Wilcoxon signed rank test), data displayed as median (interquartile range)

[#] From one-way anova for normally distributed variables, from Kruskal-Wallis test for non-normally distributed variables

[a] significantly different between MUFA and SFA diet (p<0.05 with LSD post-hoc test)

[b] significantly different between MED and SFA diet (p<0.05 with LSD post-hoc test)

[c] significantly different between MED and MUFA diet (p<0.05 with LSD post-hoc test)

Abbreviations: Alpha-2-Macroglobulin (A2M), Apolipoprotein B (Apo B), Connective Tissue Growth Factor (CTGF), Interleukin-12p70 (IL12p70), Sex Hormone Binding Globulin (SHBG), Thyroxine Binding Globulin (TBG)

DISCUSSION

We investigated the effects of replacement of SFA by MUFA in a western type diet and the effects of a full Mediterranean (MED) diet high in MUFA on PBMC gene expression and plasma protein levels. Both MUFA-rich diets decreased expression of genes involved in OXPHOS. OXPHOS is an important process to generate energy but also reactive oxygen species (ROS) are produced, which can cause oxidative damage [126, 127]. Decreases in OXPHOS gene expression in various tissues have been linked to positive as well as negative health effects in previous studies.

Studies [128] [129] showed that caloric restriction, which is considered protective against age-related decline and CVD [130], decreased expression of OXPHOS genes and oxidative stress related genes in PBMC and muscle. As a MED diet is also considered to protect against CVD and promote longevity [7, 111] and has been shown to reduce oxidative stress markers [131, 132], decreased OXPHOS might be an underlying mechanism behind these health effects. However, other studies measured decreased OXPHOS gene expression in skeletal muscle and PBMCs of type 2 diabetic subjects [133-135]. The findings in muscle led to the hypothesis that lowering of OXPHOS could cause intramuscular lipid accumulation which could lead to insulin resistance, but the association between OXPHOS and insulin resistance is still controversial. Pospisilik et al. [136] showed that a decrease of OXPHOS in muscle or liver of mice was associated with beneficial metabolic effects since it protected against diabetes. They suggested that decreased OXPHOS may be a consequence rather than a cause of insulin resistance. Expression of OXPHOS gene expression in our study was decreased after MUFA-rich diets, with concurrent improvement of serum lipid levels, but without a clear improvement in insulin sensitivity, as previously described [117]. Decreased OXPHOS gene expression in human muscle has been associated with short-term high fat feeding [137]. Our diets were relatively high in fat but since all diets contained a similar fat percentage it seems more plausible that the fat composition (i.e. high MUFA content) and not the total fat content in the diets caused the differential changes in OXPHOS gene expression.

Both MUFA-rich diets had distinct effects on expression of genes in signaling pathways. Compared to the MUFA diet, the MED diet contained additional bioactive components such as alcohol and polyphenols from red wine and extra virgin olive oil that could have affected PBMC gene expression. However, we observed that most genes were differently regulated by the MUFA diet and not by the MED diet. Other studies reported an effect of MED diet components on expression of genes related to inflammation and oxidative stress [120, 132, 138] but this was not seen in our study. When we looked more carefully at expression of inflammatory genes we observed an

unexpected high expression of some inflammatory genes such as IL1α, ILβ and TNFα in some PBMC samples. These samples were randomly distributed over the diet groups and testing days and as no correlating changes in plasma cytokine levels were observed we assumed that this effect might have been induced after blood collection. We also do not expect that this effect has influenced the outcome of our study as gene expression changes between diet groups were compared. Nevertheless, this randomly distributed high expression of inflammatory genes might have overruled possible subtle effects of the intervention diets on expression of these genes.

In plasma, both MUFA-rich diets decreased concentrations of plasma CTGF, myoglobin and ApoB. CTGF stimulates vascular smooth muscle cell proliferation and extracellular matrix production [139] and hypothetically a decrease in CTGF might lower the risk to form atherosclerotic plaques. Moreover, a decrease in myoglobin concentration might be an indication of lower oxidative stress [140]. The decrease in ApoB concentration was in accordance with the measured decrease in LDL cholesterol concentrations. The reduction in LDL, TAG and concentration of pro-atherogenic proteins with MUFA-rich diets supports previous findings of an anti-atherogenic effect of MUFA [141]. Moreover it could indicate that MUFA reduced metabolic stress, which can have consequences for systemic health and might have reduced the need for OXPHOS activity. Furthermore, within subjects in the MED diet group lower concentrations of pro-inflammatory and pro-atherogenic proteins (i.e. IL1β, Factor VII, MIP1α, SAP, TPO and VEGF) [142] [143, 144] were measured which might suggest an additional anti-atherogenic of the MED diet. The decreases in these proteins were only significant within the MED diet group but we speculate that these changes might become more pronounced after longer MED diet consumption. In previous studies also decreases in plasma Factor VII and VEGF were observed after consumption of MED diet components [121, 145, 146]. In some other studies adherence to a MED style diet was associated with decreases in vascular inflammatory markers such as IL-6, IL-7, IL-8, Inter-Cellular Adhesion Molecule 1, C Reactive Protein and Plasminogen activator inhibitor-1 [49, 120-122], while other studies [121, 147], including our study, did not observe changes in these markers. We speculate that it may be easier to lower concentrations of vascular markers in high risk subjects as included in some studies [49, 120] than in the relatively healthy subjects included in our study.

A strength of our study was the controlled food intake and high dietary compliance of the subjects. The number of subjects in our study was not very large but their food intake was accurately assessed as all meals were provided by us, in contrast to many larger studies in which dietary advice is given with a much lower control on dietary intake. Furthermore, the nutrigenomics approach applied provided us with extensive information about dietary effects on immune cells and circulating proteins. We have

used PBMCs as a model to study the systemic effects induced by consumption of diets varying in fat type. Although PBMCs are not a major cell type involved in the regulation of energy metabolism, PBMCs travel through the whole body and are exposed to all factors present in the circulation, therefore changes in OXPHOS in PBMCs may reflect systemic changes. In previous studies intake of various types of fatty acids or diets had differential effects on PBMC gene expression [24, 59, 124, 125, 148]. Based on findings in this study and in previous studies, we postulate that PBMCs can be considered as 'reporter' cells for plasma fatty acid levels and reflect the nutritional state of an individual. PBMCs are a mixture of cell populations and changes in gene expression could also be due to a shift in cell populations. Changes in cell populations were not determined and instead we had a look at changes in expression of specific monocyte or lymphocyte genes within PBMCs. Some B-cell marker genes (i.e. CD19, CD79A, CD79B) were differently expressed between the diets but it is still questionable whether these changes in gene expression are proportional to changes in subpopulations. Moreover, it is not expected that a change in B-cell population is responsible for the changes in expression of OXPHOS-related genes, as B-cells are only a minor fraction of PBMCs with a low content of mitochondria. Although it is not confirmed that the subpopulations were unchanged, the total effect on PBMC gene expression changes was caused by the diets. Because one type of fat was replaced for another in the diets it is difficult to distinguish whether the effects of the MUFA-rich diets are due to higher contents of MUFA, lower contents of SFA or a combination of both. The additional effects of the MED diet may be attributed to bioactive components in this diet but we speculate that also the larger dietary variety of the MED diet may play a role since we hypothesize that high dietary variety may improve the metabolic flexibility of subjects.

The diets induced modest changes in gene expression and plasma proteins, which was expected as MUFA and the MED food components were supplied in normal doses. Moreover the interindividual variability in response to the diets was high. Nevertheless, we think that these modest PBMC gene expression and protein changes can give information about potential mechanisms underlying the health effects of MUFA and a MED diet. Furthermore, as more information about effects of food patterns on PBMCs is emerging, the PBMC transcriptional profiles may serve as markers for dietary intake and to monitor compliance in intervention studies.

In summary, we conclude that MUFA intake as replacement of SFA in a western-type diet had similar effects by downregulating OXPHOS genes and lowering serum lipids and plasma ApoB, CTGF and myoglobin as MUFA in a MED diet. We hypothesize that replacement of SFA by MUFA increased metabolic health as reflected by these plasma changes, thereby reducing metabolic stress and OXPHOS activity in PBMCs.

Downregulation of OXPHOS may have subsequent positive health effects by lowering oxidative stress. Consumption of a full MED diet might have additional anti-atherogenic effects by lowering concentrations of pro-inflammatory plasma proteins.

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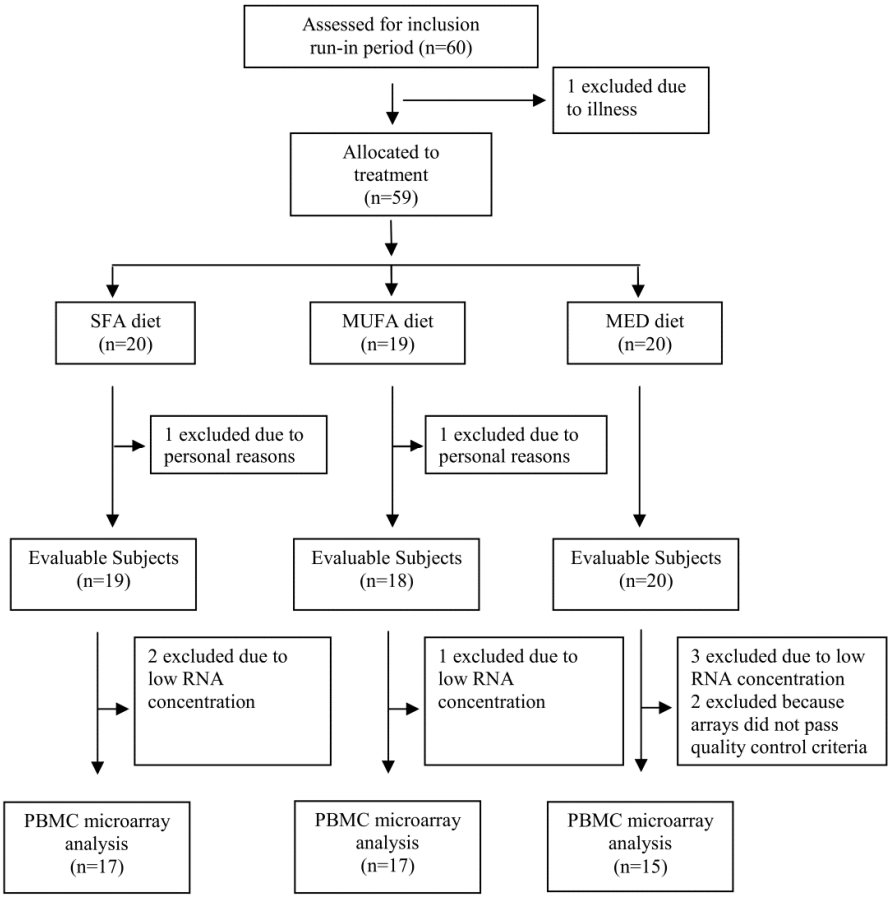
SUPPLEMENTAL DATA:



Supplemental figure 1 Flowchart of subjects included for microarray analysis
Abbreviations: Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA), Mediterranean (MED), Peripheral Blood Mononuclear Cell (PBMC)

Supplemental Table 2, plasma protein concentrations in each diet group (mean +/- standard deviation)

Protein	SFA diet		MUFA diet		MED diet	Comparison between diet groups	
	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention	*P* value [#]
Alpha-1 Antitrypsin (mg/mL)	1.99±0.22	2.00±0.26	1.96±0.32	2.13±0.48	2.09±0.42	2.07±0.36	0.231
Angiotensin Converting Enzyme (ACE) (ng/mL)	118±33	116±27	121±41	121±37	115±31	114±25	0.875
Adrenocorticotropic Hormone (ACTH) (ng/mL)	0.14±0.04	0.17±0.05*	0.19±0.2	0.20±0.15	0.24±0.19	0.22±0.16	0.095
Adiponectin (µg/mL)	3.45±2.12	3.27±1.85	4.25±2.11	4.32±2.06	4.64±3.79	4.46±3.58	0.392
Alpha-2-Macroglobulin (A2M) (mg/mL)	0.83±0.17	0.85±0.16	0.81±0.18	0.78±0.15	0.85±0.21	0.75±0.14*	**<0.05**
Alpha Fetoprotein (ng/mL)	3.71±1.17	3.77±1.23	3.95±1.36	3.93±1.41	3.68±1.44	3.59±1.40	0.715
Angiotensinogen (ng/mL)	2.49 (1.33,8.10)	1.65 (0.90,8.29)[†]	1.59 (1.32,6.73)	1.32 (0.91,3.78)	1.83 (1.12,6.12)	1.51 (1.05,8.47)	0.825
Apolipoprotein A1 (Apo A1) (mg/mL)	0.30±0.07	0.29±0.07	0.31±0.07	0.31±0.11	0.27±0.10	0.29±0.10	0.593
Apolipoprotein B (Apo B) (mg/mL)	1.09±0.27	1.12±0.29	1.1±0.17	1.03±0.20*	1.15±0.24	1.02±0.24*	**<0.005**
Apolipoprotein CIII (Apo CIII) (µg/mL)	86.4±18.1	85.6±30.2	98.1±27.8	87.6±30.0	100.4±29.7	84.3±29.4*	0.257
Apolipoprotein H (Apo H) (µg/mL)	213±43	217±45	226±44	230±54	224±41	223±43	0.840
Acylation Stimulating Protein (ASP) (ng/mL)	1704±365	1549±428	1889±380	1740±429	2014±366	1787±520*	0.793
Beta-2 Microglobulin (B2M) (µg/mL)	1.58±0.41	1.51±0.29	1.8±0.32	1.84±0.41	1.69±0.27	1.62±0.25	0.469
Betacellulin (pg/mL)	119±85	112±83	112±87	98±80	151±101	73±83	0.167
Brain-Derived Neurotropic Factor (BDNF) (ng/mL)	1.64±1.69	0.23±0.25*	1.52±1.17	0.17±0.20*	3.45±5.09	0.38±0.56*	0.241
Complement 3 (C3) (mg/mL)	0.91±0.13	0.89±0.11	1.01±0.15	1.04±0.18	0.97±0.16	0.95±0.17	0.358
Cancer Antigen 125 (CA-125) (U/mL)	9.50±5.56	7.91±6.99	8.82±8.11	9.12±8.05	10.65±9.07	8.16±5.18	0.472
Cancer Antigen 19-9 (CA-19-9) (U /mL)	6.73±6.01	6.37±5.45	7.22±8.18	7.25±7.69	6.78±5.08	7.32±5.88	0.114
Calcitonin (pg/mL)	7.97±3.4	9.59±3.90	8.85±3.95	9.51±4.05	8.41±3.83	9.01±3.01	0.579
CD40 (ng/mL)	0.62±0.08	0.56±0.08*	0.71±0.15	0.67±0.18	0.66±0.16	0.62±0.18	0.815
CD40 Ligand (CD40L) (ng/mL)	0.24±0.22	0.06±0.04*	0.22±0.17	0.05±0.03*	0.29±0.29	0.09±0.10*	0.968
Carcinoembryonic Antigen (CEA) (ng/mL)	1.48±0.88	1.53±0.80	1.32±1.58	1.47±1.97	1.59±0.66	1.51±0.57	0.140
Creatine Kinase-MB (CK-MB) (ng/mL)	0.37±0.23	0.39±0.21	0.37±0.22	0.35±0.23	0.37±0.19	0.39±0.17	0.499
Cortisol (ng/mL)	116±38	130±45	127±49	121±36	123±49	116±43	0.378
C Reactive Protein (CRP) (µg/mL)	1.25 (0.73,2.01)	1.40 (0.78,1.93)	2.80 (1.34,6.67)	3.42 (1.78,5.81)	1.32 (0.86,3.62)	1.32 (0.45,1.86)	0.128
Connective Tissue Growth Factor (CTGF) (ng/mL)	2.02±0.72	2.11±0.94	2.6±1.01	2.23±0.88*	2.09±1.24	1.75±1.11*	**<0.05**
Epidermal Growth Factor (EGF) (pg/mL)	213±117	91±42*	214±105	109±74*	225±147	95±51*	0.847
Epidermal Growth Factor Receptor (EGFR) (ng/mL)	142±35	136±37	135±39	128±36*	143±31	139±30	0.791

Epithelial cell-derived neutrophil-activating peptide 78 (ENA-78) (ng/mL)	0.88±0.75	0.12±0.14*	1.29±1.12	0.14±0.21*	1.37±1.45	0.10±0.14*	0.394
Endothelin-1 (ET-1) (pg/mL)	22.4±11.5	18.2±7.1*	25.5±13.4	21.5±11.1	24.4±11.1	23.9±10.9	0.397
Extracellular rage binding protein (EN-RAGE) (ng/mL)	5.55±3.14	5.93±4.49	9.39±8.39	6.37±4.16	9.76±6.49	7.51±7.41	0.329
Eotaxin (CCL 11) (pg/mL)	139±75	121±78	141±53	116±74*	111±108	83±79	0.858
Epiregulin (pg/mL)	26.0±24.5	28.3±31.5	17±12.5	18.5±12.4	36.1±42.4	33.7±38.7	0.610
Erythropoietin (pg/mL)	75.1±93.7	90.9±157	101±89	107±101	73.2±66.6	70.3±65.5	0.545
Fatty Acid Binding Protein (FABP) (ng/mL)	1.49±0.62	1.45±0.64	2.05±2.18	1.34±0.58	1.64±0.68	1.44±0.55*	0.258
Factor VII (ng/mL)	520±90	522±87	563±108	534±82	527±79	494±68*	0.131
Ferritin (ng/mL)	141±109	126±104*	169±102	140±94*	163±151	137±132*	0.458
Fibroblast Growth Factor Basic (bFGF) (pg/mL)	163±152	129±116*	191±208	212±223	127±56	136±79	0.105
Fibrinogen (mg/mL)	4.23±0.9	4.18±0.65	4.68±0.92	5.04±1.05	4.85±0.82	4.77±0.73	0.281
Follicle Stimulating Hormone (FSH) (ng/mL)	15.5±10.2	14.0±9.4*	16±8.9	16.6±9.2	14.2±6.5	13.7±6.7	0.075
Growth Hormone (GH) (ng/mL)	1.19±1.55	1.40±1.57	0.7±0.74	1.13±2.26	0.97±0.90	0.70±0.84	0.400
Glucagon-like peptide-1 active (GLP1 active) (pg/mL)	18.4±4.9	17.7±7.8	18.2±5	17.9±5.5	17.7±4.6	16.8±6.0	0.955
Glucagon-like peptide-1 Total (GLP1 total) (pg/mL)	83.9±37.5	84.9±39.6	73.2±34.5	77.8±39.3	73.8±33.6	72.1±27.7	0.347
Glucagon (pg/mL)	718±230	713±276	593±248	610±233	695±211	666±209	0.452
Glutathione S-Transferase (GST) (ng/mL)	1.02±0.31	1.03±0.23	1.01±0.29	0.99±0.15	0.96±0.42	0.99±0.20	0.910
Haptoglobin (mg/mL)	1.22±0.73	1.33±0.52	1.55±0.64	1.87±0.84	1.67±0.97	1.40±0.97	0.109
Heparin-Binding Epidermal Growth Factor (HB-EGF) (pg/mL)	94.9±42.8	91.9±41.8	79.8±13.5	78.3±18.4	114±66.0	105±57.2	0.464
Inter-Cellular Adhesion Molecule 1 (ICAM 1) (ng/mL)	136±27	132±18	146±64	150±63	150±47	148±46	0.480
Immunoglobulin A (IgA) (mg/mL)	1.31±0.63	1.25±0.64	1.57±0.49	1.63±0.69	1.36±0.53	1.41±0.57	0.287
Immunoglobulin E (IgE) (ng/mL)	40.5 (17.1,132)	38.3 (18.8,120)	37.4 (16.8,154)	41.9 (15.9,98.5)	63.3 (13.6,89.5)	55.8 (11.8,92.8)	0.934
Immunoglobulin M (IgM) (mg/mL)	1.06±0.65	1.03±0.69	1.17±0.59	1.15±0.55	1.13±0.60	1.11±0.55	0.980
Interleukin-10 (IL10) (pg/mL)	9.99±4.18	9.86±1.69	9.49±1.91	9.91±2.72	9.44±1.84	10.05±2.63	0.801
Interleukin-11 (IL11) (pg/mL)	41.4±30.7	43.1±31.3	80.3±153	79.9±145	67.3±79.1	54.3±46.1	0.379
Interleukin-12p70 (IL12p70) (pg/mL)	70.7±38.9	77.8±37.0	70.3±20.2	66.1±21.1	65.8±15.6	65.5±13.1	**<0.05**
Interleukin-13 (IL13) (pg/mL)	59.6±16.7	58.9±14.4	58.5±14.8	60.0±15.1	50.7±9.9	51.6±8.0	0.845
Interleukin-15 (IL15) (ng/mL)	0.32±0.12	0.31±0.12	0.3±0.09	0.28±0.09	0.31±0.08	0.31±0.08	0.693
Interleukin-16 (IL16) (pg/mL)	415±108	376±58	486±270	391±141	463±198	352±75*	0.525
Interleukin-17 (IL17) (pg/mL)	25.7±5.7	26.0±6.9	23.4±4.1	22.9±5.2	23.3±4.4	23.3±3.8	0.647
Interleukin-18 (IL18) (pg/mL)	248±68	234±73	258±72	307±188	246±89	225±83	0.130

Interleukin-1 beta (IL1b) (ng/mL)	1.86±1.51	1.20±0.60	1.56±0.67	1.23±0.52	2.85±2.87	1.21±0.55*	0.162
Interleukin-1 receptor antagonist (IL1RA) (pg/mL)	100±60.9	97.0±47.1	104±57.8	118±71.2	130±60.7	119±46.8	0.234
Interleukin-23 (IL23) (ng/mL)	0.87±0.68	0.96±1.04	0.88±1.09	0.87±1.29	0.89±0.80	0.83±0.91	0.751
Interleukin-3 (IL3) (ng/mL)	0.08±0.06	0.08±0.06	0.07±0.06	0.08±0.05	0.05±0.03	0.06±0.04	0.990
Interleukin-4 (IL4) (pg/mL)	32.5±10.9	31.8±10.7	34.5±9.2	31.5±6.1	32.7±10.9	30.5±10.6	0.560
Interleukin-5 (IL5) (pg/mL)	9.6 (6.3,13.8)	10.6 (8.1,13.8)	7.3 (6.1,12.4)	10.3 (4.6,11.9)	10.0 (4.4,13.8)	8.10 (4.4,13.8)	0.234
Interleukin-6 (IL6) (pg/mL)	0.74±0.23	0.97±0.51	2.00±2.89	1.46±0.50	1.47±0.81	1.22±0.64	0.438
Interleukin-7 (IL7) (pg/mL)	66.6±18.5	63.1±18.1	61.1±19.2	62.3±19.7	59.8±14.7	58.5±11.7	0.676
Interleukin-8 (IL8) (pg/mL)	15.5±4.5	13.2±4.2	19.3±7.4	16.4±6.8	19.8±9.8	15.3±6.0	0.334
Insulin (uIU/ml)	2.32±0.92	2.54±1.30	2.19±0.81	2.39±0.88	2.70±2.17	2.60±1.19	0.752
Leptin (ng/mL)	11.9±12.8	9.5±8.3	17.1±19.1	17.0±17.2	16.7±18.9	14.9±19.0*	0.530
Luteinizing Hormone (LH) (ng/mL)	1.8±0.7	1.79±0.78	1.79±1.43	1.91±1.68	1.41±0.55	1.50±0.66	0.499
Lipoprotein a (LPA) (µg/mL)	67 (14,116)	47 (13,128)	122 (31,431)	126 (38,481)	215 (86,500)	307 (88,500)	0.162
Monocyte Chemotactic Protein-1 (MCP1) (pg/mL)	87.3±39.2	86.0±31.1	102±35	83.0±25.1*	126±118	81.3±25.8	0.206
Macrophage colony-stimulating factor (MCSF) (ng/mL)	6.2±2.45	5.93±2.48	8.95±5.31	8.09±4.11	7.88±6.01	7.64±6.85	0.741
Macrophage Derived Chemokine (MDC) (pg/mL)	361±91	358±77	386±99	393±100	409±73	382±67	0.155
Macrophage Inflammatory Protein 1 alpha (MIP1a) (pg/mL)	57.5±11	57.6±10.3	60.8±8.1	60.1±8.8	58.1±10.2	55.5±8.3*	0.254
Macrophage Inflammatory Protein 1 beta (MIP1b) (pg/mL)	137±43	138±47	222±177	202±164*	173±50	155±51	0.121
Matrix Metalloproteinase-2 (MMP2) (ng/mL)	2049±297	2245±435	2117±416	2169±479	1994±471	2190±524*	0.364
Matrix Metalloproteinase-3 (MMP3) (ng/mL)	0.06±0.06	0.06±0.06	0.11±0.15	0.08±0.07	0.06±0.05	0.07±0.04	0.131
Matrix Metalloproteinase-9 (MMP9) (ng/mL)	104±97	92±59	94±70	60±33	161±188	151±200	0.708
Myeloperoxidase (MPO) (ng/mL)	131±55	113±30	148±52	129±45*	161±60	134±48*	0.730
Myoglobin (ng/mL)	10.0 (8.4,14.5)	12.4 (9.4,15.0)	11.8 (8.9,16.2)	10.8 (7.9,15.4)†	12.3 (9.4,16.0)	11.6 (9.0,13.2)	**<0.05**
Plasminogen activator inhibitor-1 (PAI-1) (pg/mL)	38.0±22.7	28.9±15.4*	48.7±20.4	37.6±18.2	61.4±35.3	32.1±18.7*	0.051
Pancreatic polypeptide (PP) (pg/mL)	185±79	162±50	244±179	213±96	161±90	150±48	0.842
Prostatic Acid Phosphatase (PAP) (ng/mL)	0.22 (0.20,0.34)	0.25 (0.20,0.39)	0.25 (0.20,0.30)	0.25 (0.21,0.29)	0.28 (0.23,0.39)	0.26 (0.25,0.34)	0.272
Pregnancy-associated plasma protein A (PAPPA) (mIU/mL)	0.04±0.01	0.04±0.01	0.04±0.01	0.03±0.01	0.04±0.02	0.04±0.02	0.563
Platelet-derived growth factor (PDGF) (pg/mL)	2134±1096	2344±1048	1869±1158	2205±862	2593±2216	2213±908	0.527
Progesterone (ng/mL)	5.05 (2.79,6.12)	4.58 (3.06,6.22)	4.27 (2.97,4.78)	3.61 (2.94,4.02)†	4.61 (3.02,4.88)	4.49 (3.28,6.91)	0.154

Prolactin (ng/mL)	14.3±4.8	14.9±5.4	17.2±3.7	15.4±3.8	14.3±5.7	13.7±4.7	0.217
Prostate Specific Antigen (PSA) (ng/mL)	0.11 (0.03,0.67)	0.10 (0.04,0.58)	0.07 (0.04,1.04)	0.07 (0.04,1.04)	0.07 (0.03,0.57)	0.05 (0.04,0.43)	0.186
Peptide YY (PYY) (pg/mL)	63.6±26.4	72.4±39.8	60.1±39.4	67.0±36.4	60.0±47.9	58.5±37.4	0.456
Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) (ng/mL)	6.50±5.70	1.11±1.20*	7.11±4.83	0.90±0.79*	13.07±14.76	1.87±2.58*	0.205
Resistin (ng/mL)	3.34±1.1	3.25±1.16	3.35±0.85	3.35±1.00	3.48±0.77	3.36±0.61	0.753
Serum Amyloid P (SAP) (µg/mL)	18.4±4.7	17.9±4.2	19.8±4.8	20.2±5.3	20.8±4.7	18.8±5.6*	0.080
Stem Cell Factor (SCF) (pg/mL)	219±69	208±62	231±67	196±77*	223±90	171±65*	0.078
Serum glutamic oxaloacetic transaminase (SGOT) (µg/mL)	15.7±2.5	15.6±2.3	15.3±2.7	16.6±3.1	16.6±3.1	16.0±2.3	0.179
Sex Hormone Binding Globulin (SHBG) (nmol/L)	51.7±28.5	48.0±23.9*	39.8±15.9	41.4±18.9	36.5±15.0	36.5±14.9	**<0.05**
Thyroxine Binding Globulin (TBG) (µg/mL)	52.8±14.9	51.7±12.3	54.6±9	57.8±10.5*	57.5±16.6	54.8±15.0*	**<0.05**
Tenascin C (ng/mL)	1228±513	1248±508	1434±809	1415±674	1264±1003	1180±778	0.595
Testosterone (ng/mL)	1.74±1.30	1.54±1.10*	1.57±1.12	1.49±1.10	1.50±1.19	1.45±1.24	0.383
Tissue Factor (TF) (ng/mL)	0.71±0.37	0.69±0.35	0.66±0.28	0.71±0.30	0.60±0.30	0.58±0.29	0.204
Tissue inhibitor of metalloproteinase 1 (TIMP1) (ng/mL)	80.5±13.5	73.3±9.5*	83.7±11.2	75.5±12.9*	90.2±24.9	71.1±8.7*	0.086
Tumour necrosis factor alpha receptor type II (TNFaRII) (ng/mL)	3.12±0.68	3.09±0.37	3.85±0.94	3.95±0.95	3.63±1.04	3.55±0.79	0.603
Tumour necrosis factor alpha (TNFa) (pg/mL)	7.27±1.33	7.12±1.28	8.12±1.86	8.01±2.40	8.08±3.10	7.20±2.66	0.477
Thrombopoietin (TPO) (ng/mL)	1.28±0.93	1.07±0.73	1.5±1.36	1.14±0.79	1.31±0.83	0.81±0.78*	0.571
Thyroid Stimulating Hormone (TSH) (µIU/mL)	1.97±0.83	2.10±0.84	5.07±9.55	5.49±10.87	2.12±1.04	2.10±1.17	0.441
Thrombospondin-1 (THBS1) (ng/mL)	7182 ±5934	1104 ±1428*	7066 ±5430	733 ±825*	12327 ±13609	1981 ±3338*	0.382
Vascular cell adhesion molecule-1 (VCAM1) (ng/mL)	542±113	534±84	553±104	590±120*	577±115	593±123	0.131
Vascular endothelial growth factor (VEGF) (pg/mL)	594±155	556±119	635±118	612±98	632±166	573±119*	0.459
von Willebrand Factor (vWF) (µg/mL)	27±10.7	26.2±8.3	36.1±11.8	38.7±14.1	28.8±210.7	25.2±8.1	0.201

* significantly different from baseline (p<0.05 in paired t-test), data displayed as mean ± standard deviation (all such values)

† significantly different from baseline (p <0.05 in Wilcoxon signed rank test), data displayed as median (interquartile range) (all such values)

From one-way anova for normally distributed variables, from Kruskal-Wallis test for non-normally distributed variables

CHAPTER 4

Plasma protein profiling reveals protein clusters related to BMI and insulin levels in middle-aged overweight subjects

SJ van Dijk, EJM Feskens, AG Heidema, MB Bos, O van de Rest,
JM Geleijnse, LCPGM de Groot, M Müller and LA Afman

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ABSTRACT

Background: Biomarkers that allow detection of the onset of disease are of high interest since early detection would allow intervening with lifestyle and nutritional changes before the disease is manifested and pharmacological therapy is required.
Objective: Our study aimed to improve the phenotypic characterization of overweight but apparently healthy subjects and to identify new candidate profiles for early biomarkers of obesity-related diseases such as cardiovascular disease and type 2 diabetes.
Design: In a population of 56 healthy, middle-aged overweight subjects Body Mass Index (BMI), fasting concentration of 124 plasma proteins and insulin were determined. The plasma proteins are implicated in chronic diseases, inflammation, endothelial function and metabolic signaling. Random Forest was applied to select proteins associated with BMI and plasma insulin. Subsequently, the selected proteins were analyzed by clustering methods to identify protein clusters associated with BMI and plasma insulin. Similar analyses were performed for a second population of 20 healthy, overweight older subjects to verify associations found in population I.
Results: In both populations similar clusters of proteins associated with BMI or insulin were identified. Leptin and a number of pro-inflammatory proteins, previously identified as possible biomarkers for obesity-related disease, e.g. Complement 3, C Reactive Protein, Serum Amyloid P, Vascular Endothelial Growth Factor clustered together and were positively associated with BMI and insulin. IL-3 and IL-13 clustered together with Apolipoprotein A1 and were inversely associated with BMI and might be potential new biomarkers.
Conclusion: We identified clusters of plasma proteins associated with BMI and insulin in healthy populations. These clusters included previously reported biomarkers for obesity-related disease and potential new biomarkers such as IL-3 and IL-13. These plasma protein clusters could have potential applications for improved phenotypic characterization of volunteers in nutritional intervention studies or as biomarkers in the early detection of obesity-linked disease development and progression.

INTRODUCTION

Cardiovascular disease (CVD) and type 2 diabetes (T2DM) are common disorders affecting millions of people worldwide. Evidence is accumulating that chronic low-grade inflammation plays a role in the development of both diseases [149, 150]. Increased plasma levels of several pro-inflammatory proteins and decreased levels of anti-inflammatory proteins have been observed in subjects with obesity and obesity-related diseases such as CVD and T2DM [151-154].

Certain pro-inflammatory plasma proteins are used as diagnostic biomarkers for disease state but specific plasma proteins may also be used as biomarkers for early state in the development of a disease. Such an improved pre-disease diagnostic would allow intervening with relatively mild strategies such as lifestyle interventions with specific dietary regimes and increased physical activity in contrast to pharmacological therapy required once the disease is manifested. Identification of biomarkers that allow detection of the onset of disease will help in prevention of the disease. Plasma proteins might be good candidates as they circulate throughout the whole body, thereby reflecting total body metabolic and inflammatory status. Moreover, blood can be easily obtained from human subjects and therefore plasma proteins can be easily measured for screening purposes.

So far, in most studies that investigated the use of plasma proteins as biomarkers only a few plasma proteins were measured. However, the etiology of diseases such as CVD and T2DM is complex and the measurement of multiple biomarkers will provide additional information about the individual phenotype and health status as compared with measurement of a single biomarker [155, 156]. Recent technological advances such as multiplex immunoassays allow for the measurement of over hundred proteins at a time in one small plasma sample. Identification of biomarker profiles in such large protein datasets requires advanced statistical analyses. Random Forest (RF) has shown to be suitable for analysis of complex data sets as derived from proteomics analysis [157, 158]. RF is a technique that can prioritize and select from a large number of variables a set of variables that is likely to be related to the outcome of interest. Furthermore, in the prioritization and selection process, it provides a way to take interactions between proteins into account [159, 160]. The proteins that are selected by RF can subsequently be analyzed by clustering methods, offering the opportunity to identify clusters of proteins that are associated with different health outcomes.

Our study aimed to improve the phenotypic characterization of overweight but apparently healthy subjects and to identify new candidate profiles for early biomarkers of obesity-related diseases such as CVD and T2DM.

SUBJECTS AND METHODS

Subjects

Two populations were included in this study; population I was the primary study population of interest and population II was a smaller population used for verification of the results found in population I.

Population I consisted of 56 healthy men and women who participated in a controlled feeding trial [117]. Subjects included were aged 40-65 years with a BMI $\geq$ 25 kg/m^2 or a waist circumference $\geq$ 94 cm for men and $\geq$ 80 cm for women. Excluded were hypercholesterolemic subjects (fasting total cholesterol $\geq$ 8 mmol/L) and subjects with non-treated diabetes mellitus (according to WHO criteria) as measured by an oral glucose tolerance test during screening. Other exclusion criteria were the use of serum lipid or blood pressure lowering medication.

Population II consisted of 20 healthy, independently living elderly men and women. This population is a subgroup of the population participating in the study of Van de Rest et al. [161]. Subjects included were aged >65 years without depression, dementia or serious liver disease. This population was chosen because subjects were healthy and had average BMI and insulin values comparable to those from population I.

Both studies were approved by the Medical Ethics Committee of Wageningen University and all subjects gave written informed consent.

Plasma proteins

In population I, concentrations of 124 proteins, including insulin, were measured in fasting plasma by quantitative multiplex immunoassay based on Luminex xMAP technology (Rules Based Medicine Inc, Austin, Texas, USA) according to the procedure described by Domenici et al. [162] [163]. For each multiplex, both calibrators and controls were included on each microtiter plate. 8-point calibrators were run in the first and last column of each plate and 3-level controls were included in duplicate. Testing results were determined first for the high, medium and low controls for each multiplex to ensure proper assay performance. Unknown values for each of the analytes localized in a specific multiplex were determined using 4 and 5 parameter, weighted and non-weighted curve fitting algorithms included in the data analysis package. The plasma samples were run in duplicate and data reported as concentrations (average of two independent measures), together with data for the least detectable dose (LDD). Any value above the LDD will possess coefficients of variation (CV) less than 20%. Rules-Based Medicine's Multi-Analyte Profiles have been validated to Clinical Laboratory Standards Institute guidelines.

The set of proteins present on the assay consists of factors that are implicated in chronic diseases, inflammation, endothelial function and metabolism. In table S1 (http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0014422#s5) all proteins measured are listed.
Before analysis 16 out of 124 proteins (table S1) were removed from the dataset as the concentrations of these proteins were below the detection limit in more than half of the samples. For the remaining 108 proteins, values below the detection limit were replaced by 0.1*Least Detectable Dose. As IL-6 was one of the removed proteins and IL-6 is an important factor for obesity and diabetes [154], this protein was separately measured by high-sensitive enzyme immunoassay (Human IL-6 Quantikine HS ELISA Kit, R&D Systems, Abingdon, United Kingdom). Apo lipoprotein B (ApoB) was not included in the Human Multi-Analyte Profiles, and ApoB levels were additionally measured on a Hitachi 912 autoanalyser (Roche, Lelystad, The Netherlands) using a commercially available kit (Roche cat. nr.1551779). In population II, the plasma concentrations of 107 out of the 124 proteins measured in population I, were determined using multiplex immunoassay (Rules Based Medicine Inc, Austin, Texas, USA) (table S1). In total, 110 proteins were included in the analysis for population I and 90 proteins were included in the analysis for population II (online figure S1, http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0014422#s5)

Statistical analyses

Univariate analyses
The association between individual protein concentrations, BMI and plasma insulin concentration was calculated by univariate analysis. The statistical package PASW (version 17.0; SPSS, Chicago, IL) was used for the univariate analysis. Since the distribution of several variables was slightly skewed in the population, Spearman correlation coefficients were calculated for the association between protein concentrations, BMI and insulin concentrations.

Random Forest
Random Forest (RF) was used to provide a ranking in the importance of proteins in their relationship with BMI as well as with plasma insulin concentrations, taking possible interactions between proteins into account. The R package randomForest (R-package randomForest, http://cran.r-project.org/), which is based on the original FORTRAN code from Breiman et al. [159] was used for the analysis (www.stat.berkeley.edu/~breiman/RandomForests/).

In RF a group of tree-based models (the forest) is used to rank the proteins with an important contribution to BMI or insulin values [160, 164]. Each tree starts with the total data set, which is split into smaller and more homogeneous groups to fit models for predicting the outcome from the measured proteins. Within the forest, different trees are obtained by bootstrap sampling and random subset selection.
Importance of proteins in association with the outcome of interest is defined by a measure referred to as the importance index, I_m. For each protein, this I_m is obtained by comparing the predictive performance of the forest for all proteins with the predictive performance of the forest in which the values of the protein are randomly permuted in the trees for the left-out observations. Larger differences in the predictive performance give a larger I_m, indicating that the protein is more important. By permuting the values for one protein, not only the effect of this protein is taken into account, but also all possible interactions of this protein with other proteins. Interactions between proteins increase the I_m for each of the proteins that are part of the interaction. Thus, in the ranking of proteins by their importance RF takes interactions between proteins into account.
To perform the RF analyses we used the scaled mean decrease in prediction accuracy. To obtain stable estimates of the I_m and to capture as many important interactions as possible, the analyses were performed with a large number of trees (40,000). I_m was used as measure to rank the proteins. We chose not to apply a FDR estimation of the Im scores, because FDR estimation of importance scores derived by tree-based approaches usually overestimates the real FDR it can lead to an unreliable selection of a subset of variables [165].
For RF analysis a threshold value of significance does not exist. In this study a threshold was set at an I_m of 5 and only proteins with an $I_m > 5$ were considered for subsequent cluster analyses. We chose for this liberal threshold to avoid the possibility of leaving out proteins that might be of importance in relation to BMI and insulin.

Clustering of the proteins
The program MultiExperimentViewer, version 4.3 was used for hierarchical clustering and visualization of the data [166]. Hierarchical clustering organizes the data into a binary tree that groups similar elements together. Proteins, BMI and insulin were clustered based on their Spearman correlation coefficients to select groups of proteins with high correlation with BMI or insulin and with each other. Besides clustering of the proteins with BMI and proteins with insulin based on their correlation coefficients also individual protein profiles were clustered based on similarities in protein concentrations. To compare the individual protein concentrations, z-scores were

calculated for each individual protein $\left(\frac{x - \mu}{\sigma} \right)$. To compare the association of the identified clusters of proteins and BMI to the association of single traditional biomarkers and BMI regression analysis was performed using the statistical package PASW (version 17.0; SPSS, Chicago, IL).

Pathway analysis

Ingenuity Pathways Analysis, version 8.7 (Ingenuity® Systems, www.ingenuity.com) was used to identify connections between proteins and canonical pathways and diseases that were most significant to the data. Proteins were entered in Ingenuity based on their Swiss Prot ID and only connections, both direct and indirect, between proteins for humans and human primary cells were considered in the analysis.

RESULTS

Baseline characteristics

Characteristics of the two study populations used in the analysis are displayed in table 1. BMI, waist circumference, insulin levels and percentage smokers were comparable in the two populations and age was significantly higher (13.7 ± 1.1) in population II. Plasma protein concentrations for both populations are displayed in online table S2 (http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0014422#s)

Table 1 Characteristics of the study populations

	Population I (n=56)	Population II (n=20)
Sex (m/f)	23 / 33	10 / 10
Age (years)*	56.4 ± 6.7	70.1 ± 3.1
BMI (kg/m^2)	27.4 ± 5.0	28.3 ± 5.4
Smokers (%)	11 %	10 %
Waist circumference (cm)	96.9 ± 13.2	99.0 ± 13.8
Insulin (pmol/L)	16.11 ± 8.84	14.24 ± 8.05

Data is displayed as mean ± standard deviation

* Significantly different between the populations, $p<0.05$

Associations of proteins with BMI

In population I the RF I_m of 20 proteins was above 5 and these proteins were considered to be associated with BMI (table 2). Using univariate analysis, 14 out of these 20 selected proteins correlated significantly with BMI (table 2). For these 20 proteins and BMI mutual correlation coefficients were calculated. Based on these correlation coefficients a correlation matrix was constructed in which the proteins and BMI were clustered by similarity in their correlations (figure 1A). Using this approach, three clusters of proteins associated with BMI could be identified; cluster 1 and 3 were positively associated with BMI while cluster 2 was negatively associated with BMI. Cluster 1 showed robust associations with BMI and contains proteins highly positively associated with BMI and with each other and included insulin, leptin, Complement 3 (C3), Interleukin 6 (IL-6), C Reactive Protein (CRP), Plasminogen Activator Inhibitor (PAI-1), Serum Amyloid P (SAP) and Vascular Endothelial Growth Factor (VEGF). Cluster 2 also showed robust associations with BMI and the included proteins were inversely associated with BMI and positively with each other (figure 1A) and contained the proteins Apolipoprotein A1 (ApoA1), Cancer Antigen 19-9 (CA 19-9), Eotaxin, IL-3 and IL-13. The third cluster includes proteins that were positively associated with BMI and each other but most of these associations were less pronounced than in clusters 1 and 2.

Table 2 Association of proteins with BMI in the two populations.

	Population I (n=56)		Population II (n=20)	
Protein	I_m RF	Spearman correlation	I_m RF	Spearman correlation
1. Leptin	71.8	0.47 **	39.4	0.76 **
2. MCSF	49.1	0.18	n.d.	n.d.
3. IL-3	42.8	-0.43 **	11.7	-0.67 **
4. Insulin	35.4	0.48 **	51.4	0.74 **
5. Apo A1	33.0	-0.48 **	24.4	-0.66 **
6. IL-13	25.7	-0.46 **	26.1	-0.57 **
7. PAI-1	19.7	0.48 **	22.7	0.63 **
8. Eotaxin	17.2	-0.40 **	<5	-0.29
9. CA19-9	16.2	-0.06	<5	0.30
10. CRP	15.0	0.46 **	10.4	0.60 **
11. MDC	14.4	0.20	<5	0.01
12. Resistin	14.1	0.27 *	<5	0.37
13. IL-6	11.4	0.42 **	n.d.	n.d.
14. Apo B	11.1	0.20	n.d.	n.d.
15. SAP	8.6	0.38 **	34.6	0.70 **
16. C3	8.5	0.42 **	36.5	0.48 *
17. MIP1b	7.5	0.25	<5	0.31
18. VEGF	6.1	0.38 **	22.8	0.59 **
19. FABP	5.4	0.20	<5	0.11
20. LPA	5.2	0.26 *	<5	-0.11

* $p < 0.05$, ** $p < 0.005$

Abbreviations: not determined (n.d.), Importance Index Random Forest (I_m RF), Macrophage colony-stimulating factor (MCSF), Apolipoprotein A1 (ApoA1), Plasminogen Activator Inhibitor-1 (PAI-1), Cancer Antigen 19-9 (CA 19-9), C Reactive Protein (CRP), Macrophage Derived Chemokine (MDC), Apolipoprotein B (ApoB), Serum Amyloid P (SAP), Complement 3 (C3), Macrophage Inflammatory Protein-1 beta (MIP1b), Vascular endothelial growth factor (VEGF), Fatty Acid Binding Protein (FABP), Lipoprotein A (LPA).

In population II 22 proteins were associated with BMI, based on RF I_m above 5, of which ten proteins were also associated with BMI in population I (table 2 and figure S1). A correlation matrix of these ten proteins and BMI was made for population II. Clustering of the proteins in population II was similar to the clustering in population I (figure 1A and 1B). The protein clusters 1 and 2, which showed robust associations in population I, were also associated with BMI in population II. The weaker associations of protein cluster 3 could not be confirmed. Associations in population II were more robust compared to associations in population I.

Regression analysis to compare the association of the identified clusters of proteins and BMI to the association of single traditional biomarkers such as CRP and IL-6 showed that the explained variance was higher when all proteins of cluster 1 were included in the model compared to when only IL6 or CRP or a combination of both were included. For BMI, the proportion of variance explained was 16.3% for IL-6 alone, 19.4% for CRP alone, 22.0% for CRP and IL-6 combined, and 32.3% when all proteins from cluster 1 were included in the model. For insulin, cluster 1 explained 25.6% of the variance, compared to 8.3% by CRP alone, 11.8% by IL-6 alone, and 12.0% by CRP and IL-6 combined.

Out of the cluster analysis we selected the highly BMI-associated proteins from cluster 1 to plot individual plasma profiles (figure 2). Subjects with similar plasma protein concentrations were clustered and their BMI values were subsequently displayed. Figure 2 shows that, as expected, in general persons with higher BMI have higher concentrations of the selected proteins. However, a few persons with BMI values <25 kg/m^2 had high plasma levels of these proteins and a few persons with BMI values >30 kg/m^2 had low plasma protein levels

Association of proteins with insulin concentration

The association between protein profiles and fasting insulin concentration was investigated using the same approach as for BMI. In population I, RF analysis identified 20 proteins that were considered to be associated with insulin concentration (table 3). Using univariate analysis, ten of these proteins significantly correlated with insulin concentration. Hierarchical clustering of the 20 selected proteins and insulin based on correlation coefficients resulted in the formation of four separate protein clusters (figure 3A). The proteins forming cluster 1, 2 and 3 were all positively associated with insulin concentration and the proteins in the fourth cluster were negatively associated with insulin.

In population II, 9 out of the 20 selected proteins in population I were associated with plasma insulin concentrations (table 3, figure 3B and figure S1). As for BMI associations with insulin in population II were more robust compared to associations with insulin in population I.

Table 3 Association of proteins with fasting plasma insulin in both populations

	Population I (N=56)		Population II (N=20)	
Protein	Im RF	Spearman correlation	Im RF	Spearman correlation
1. SAP	42.9	0.518**	25.8	0.579*
2. Leptin	32.2	0.333*	23.3	0.695**
3. PAP	29.2	0.411**	< 5	-0.165
4. MIP1b	28.8	0.376**	< 5	0.104
5. C3	18.5	0.379**	29.0	0.621**
6. IL-7	16.9	0.077	< 5	-0.492*
7. FABP	16.8	0.416**	< 5	0.099
8. ASP	16.0	0.383**	< 5	0.220
9. VEGF	11.1	0.296*	21.3	0.590*
10. CK_MB	10.0	0.040	41.8	-0.614*
11. TNFα	9.9	0.250	< 5	0.340
12. MPO	9.0	0.257	30.4	0.450*
13. IL-6	8.0	0.363**	n.d.	n.d.
14. PP	7.8	-0.171	< 5	-0.219
15. Apo CIII	7.5	-0.240	< 5	-0.072
16. ENA-78	7.2	0.082	5.5	0.520*
17. Factor VII	6.8	0.243	10.9	0.460*
18. IL-8	6.1	0.149	< 5	0.155
19. IL-18	5.8	0.230	< 5	-0.169
20. CRP	5.1	0.317*	36.6	0.723**

* $p<0.05$, ** $p<0.005$

Abbreviations: Importance factor Random Forest analysis (Im RF), not determined (n.d), Serum Amyloid P (SAP), Prostatic Acid Phosphatase (PAP), Macrophage Inflammatory Protein-1 beta (MIP1b), Complement 3 (C3), Fatty Acid Binding Protein (FABP), Acylation Stimulating Protein (ASP), Vascular endothelial growth factor (VEGF), Creatine Kinase-MB (CK-MB), Tumor Necrosis Factor alpha (TNFα), Myeloperoxidase (MPO), Pancreatic Polypeptide (PP), Apolipoprotein CIII (Apo CIII), Epithelial neutrophil-activating peptide 78 (ENA-78), C Reactive Protein (CRP)

Pathway analysis

An overview of all clusters of proteins and interactions between the single proteins selected by RF is displayed in figure 4 (page 71). This figure also shows the top 5 most significant pathways and diseases for the BMI- and insulin-associated proteins. Acute phase response signaling was the most significant pathway for BMI-associated proteins and was also significant for insulin-associated proteins.

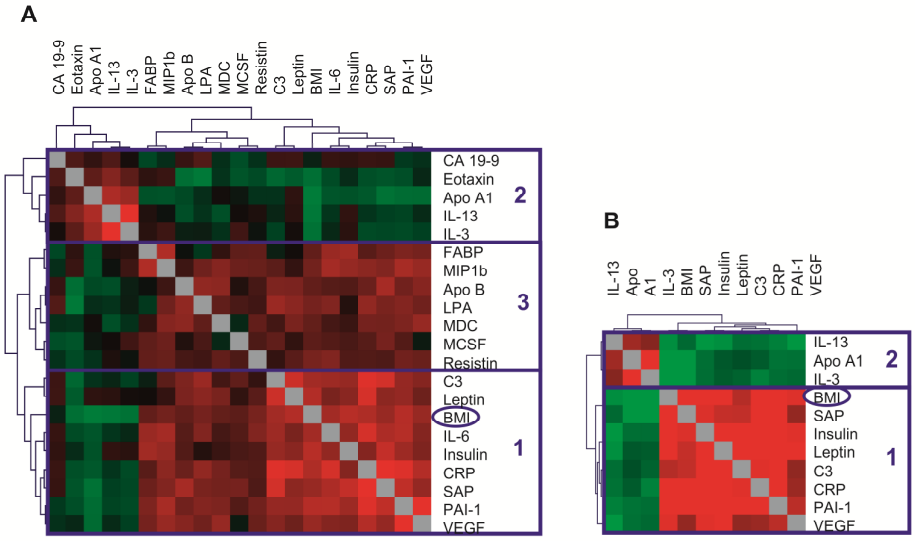

Figure 1. Correlation matrix of selected proteins associated with BMI in population I (A) and II (B).
Correlation coefficients are displayed on a color scale, ranging from -0.6 (green) to 0.6 (red). For population I (A) 20 proteins selected by RF to be associated with BMI are included. This group of proteins was divided in three clusters based on similarity in the association with BMI and with each other. For population II (B) 10 proteins selected by RF and also associated with BMI in population I are included. This group of proteins was divided in two clusters based on similarity in the association with BMI and with each other.

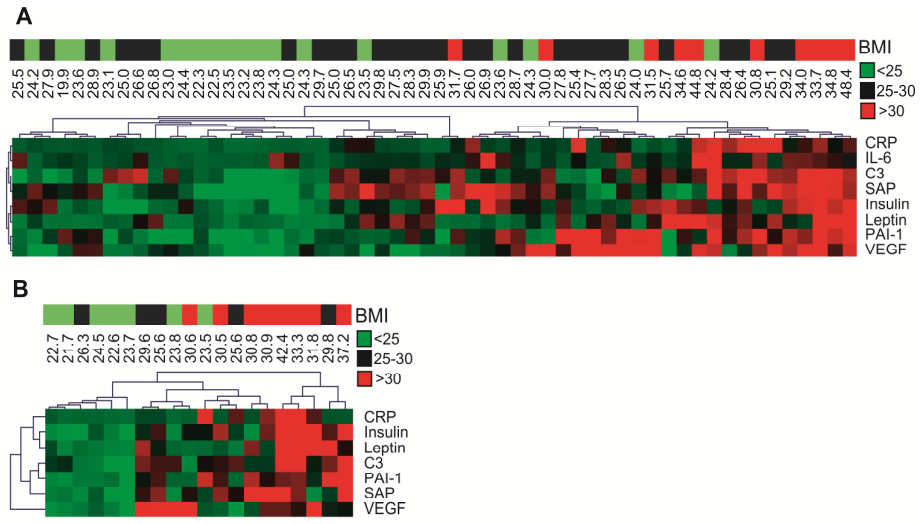

Figure 2. Clustering of personal profiles of robustly positively BMI-associated proteins in population I (A) and II (B).

Protein values are displayed on a color scale; red indicates higher values than group average, green indicated lower values than group average. The personal BMI values are displayed on a separate color scale; green for BMI<25, black for BMI 25-30 and red for BMI>30. Only robustly positively BMI-associated proteins from cluster 1, as identified by RF and cluster analysis, were included in this figure.

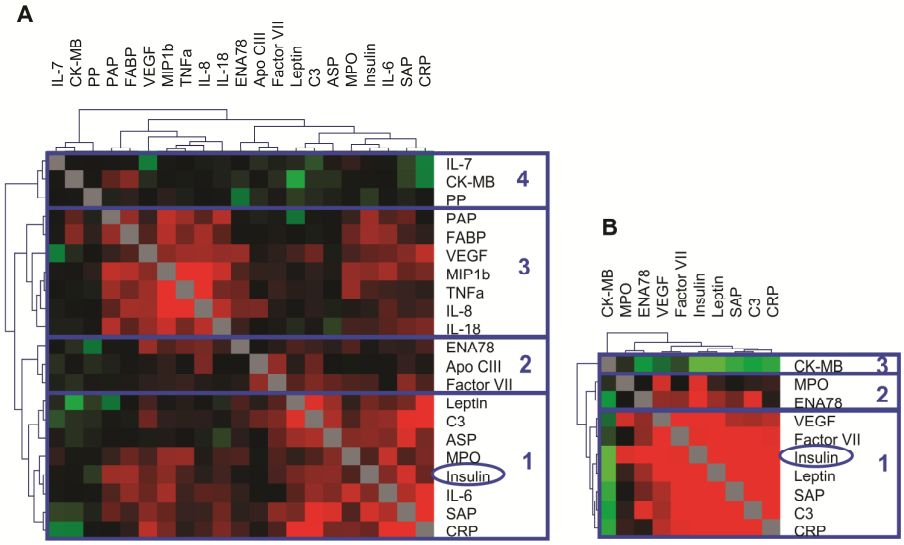

Figure 3. Correlation matrix of selected proteins associated with insulin in population I (A) and II (B).
Correlation coefficients are displayed on a color scale, ranging from -0.6 (green) to 0.6 (red). For population I (A) 20 proteins selected by RF to be associated with fasting plasma insulin are included. This group of proteins was divided in four clusters based on similarity in the association with insulin and with each other. For population II (B) 9 proteins selected by RF and also associated with BMI in population I are included. This group of proteins was divided in three clusters based on similarity in the association with insulin and with each other.
Abbreviations: Creatine Kinase-MB (CK-MB), Pancreatic Polypeptide (PP), Prostatic Acid Phosphatase (PAP), Fatty Acid Binding Protein (FABP), Vascular endothelial growth factor (VEGF), Macrophage Inflammatory Protein-1 beta (MIP1b), Tumor Necrosis Factor alpha (TNFα), Epithelial neutrophil-activating peptide 78 (ENA-78), Apolipoprotein CIII (Apo CIII), Complement 3 (C3), Acylation Stimulating Protein (ASP), Myeloperoxidase (MPO), Serum Amyloid P (SAP), C Reactive Protein (CRP)

DISCUSSION

Elevated plasma levels of several pro-inflammatory proteins are related to the development of obesity-linked diseases, in particular to T2DM and CVD [152, 167-169]. In the current study we observed associations of clusters of pro- and anti-inflammatory proteins with BMI and insulin in a presumably healthy population. These BMI- and insulin-associated protein clusters may serve as biomarkers for a pre-disease state of people at risk to develop CVD and T2DM. The protein clusters could possibly be used to improve individual disease risk prediction and help in the design of personalized strategies to prevent disease as early as possible.

The protein cluster showing the most robust positive association with BMI contained a number of pro-inflammatory proteins of which several are involved in the acute phase response, such as CRP, IL-6, C3 and SAP. These and other proteins (i.e. ASP, MPO, PAI-1 and VEGF) included in this cluster and in cluster 1 that was associated with insulin were previously found to be increased in subjects with insulin resistance, CVD, or both [143, 151, 168, 170-178]. From prospective studies evidence has accumulated that increased levels of C3 and CRP can predict T2DM and coronary events and could be candidate biomarkers for a pathological state preceding the ultimate disease [151, 156, 172, 173]. We hypothesize that the proteins clustering together with CRP and C3 could be similar type of biomarkers. Moreover, Macrophage Colony-Stimulating Factor (MCSF) which was highly positively associated with BMI was discovered to be a prognostic marker of cardiovascular events in patients with chronic coronary artery disease [179]. We speculate that MCSF may also be an early biomarker for cardiac disease in healthy subjects.

The positive correlations of BMI with leptin and other adipose-tissue derived proteins, as seen in our study, supports the view of adipose tissue as an important source of immune-related proteins [180]. However, besides adipose tissue-derived proteins also proteins produced by the liver, endothelial cells, immune cells and lipoproteins were associated with BMI and insulin in our healthy subjects. This indicates that low-grade inflammation in the early disease state is not only an adipose tissue-specific effect but that also organ cells increase their secretion of inflammatory proteins with increasing BMI or plasma insulin levels.

A small cluster of proteins (ApoA1, CA19-9, Eotaxin, IL-3 and IL-13) was negatively associated with BMI. Plasma levels of tumor marker CA19-9 and ApoA1, the major protein component of plasma HDL, were reported to be lower in obesity [181, 182]. Less is known about the negative association of BMI with Th2 cytokines IL-3 and IL-13 and Eotaxin, which are correlated with each other and closely clustered [183-185]. Concentrations of IL-3 and IL-13 were on average very low in the populations so we

should be careful with the interpretation of this data. Nevertheless we observed associations of IL-3 and IL-13 with BMI in both study populations, therefore these proteins might be considered as potential new early biomarkers for obesity-related diseases.

Associations between strongly associated protein clusters 1 and 2 with BMI and insulin in the primary study population were confirmed in the second population. Weaker associations were not all confirmed which could be attributable to the lower number of subjects in population II and the fact that less proteins were measured reducing the chance of finding protein interactions with RF. However, the strong associations found in population I were even more pronounced in population II. The latter population was significantly older but concentrations of individual plasma pro-inflammatory proteins were not higher. Maybe an elevated BMI or insulin concentration in elderly subjects is directly linked to an increase in pro-inflammatory protein secretion while this is not always the case in younger subjects who may display a more flexible metabolic phenotype in handling changes in BMI or insulin concentration.

Levels of individual proteins were not extremely elevated in our study subjects, but based on protein profiles subjects may be differentially classified with lower or higher risk to develop a more pathological phenotype. Despite the fact that the risk profile was BMI-related in the whole study population, there were a few subjects with low BMI also having this risk profile and a few subjects with high BMI not having the profile. Besides BMI, waist-to-height ratio can be used as a determinant for obesity-related health risk. When the same analyses were performed for the association of proteins with waist-to-height ratio similar results as for BMI were found. Our findings support the recent ideas of using a ‘multimarker’ approach, i.e. measuring multiple plasma proteins in instead of single biomarkers or only BMI to increase the prognostic value for individual disease risk [156, 186, 187].

Still, the measurement of multiple proteins makes data analysis complex and calls for more advanced methods of data analysis. Using RF we were able to identify associations of proteins with BMI or insulin that were not observed when associations of each protein were analyzed separately, but combined with other proteins may be of relevance. For RF we chose a liberal threshold for selection of proteins associated with BMI or insulin, which increases the risk of selecting proteins that are not related to the outcome of interest. However, using this threshold, we observed a high overlap between the results from RF and univariate analysis. Furthermore, we applied this analysis to two independent populations to replicate and verify the associations of protein clusters with BMI and insulin. In our view, this approach has increased the reliability of our results.

The results of our study are based on a single measurement of plasma proteins that provides a "snapshot" of the actual health status. Whether it can be used in predicting long-term health and inflammatory status requires more long-term studies. Furthermore, our primary study population consisted of a relatively low number of subjects. However, we have observed associations of proteins with BMI or insulin that are physiologically relevant and were found in epidemiological and clinical studies [143, 151, 153, 154, 156, 168, 170, 177, 178]. Moreover, we were able to confirm the most robust associations in a second, even smaller population consisting of subjects from a different age group.

With our study it is not possible to draw conclusions about the causal relationship between elevated concentrations of the selected proteins and occurrence of disease. Further prospective studies with clinical end points are needed to determine whether the protein clusters found in this study could be used as reliable biomarkers for early identification of persons at risk for T2DM and CVD.

Our study aimed at identifying new leads for clusters of early biomarkers of disease by using plasma protein profiling in healthy subjects. In healthy subjects we identified clusters of proteins associated with BMI and insulin that included previously identified biomarkers for obesity-related disease risk and potential new biomarkers for which an association with disease is not well-established. We showed that plasma protein profiling allows a more subtle phenotypic characterization and differentiation of people with otherwise similar phenotypical features such as BMI or insulin levels. This could be of great value for dietary and pharmacological intervention studies where subgroups of volunteers with matching phenotypes could be included in order to improve the power of such interventions. Improved individual risk assessment and classification of subjects may ultimately lead to a more tailored and adequate intervention either by pharmacology or changes in lifestyle.

ACKNOWLEDGMENTS

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Figure 4 (next page). BMI- and insulin-associated proteins, their interactions and pathways and diseases in which they are involved.

All defined clusters of BMI and insulin-associated proteins are displayed, in red the positively associated clusters and in green the negatively associated clusters. In dark red or green BMI and insulin-associated proteins in both populations are shown, in light red and light green only the BMI and insulin-associated proteins in population I. Connections between proteins and the top 5 canonical pathways and diseases relevant to the data are shown.

Abbreviations: Triggering Receptor Expressed on Myeloid cells 1 (TREM1)

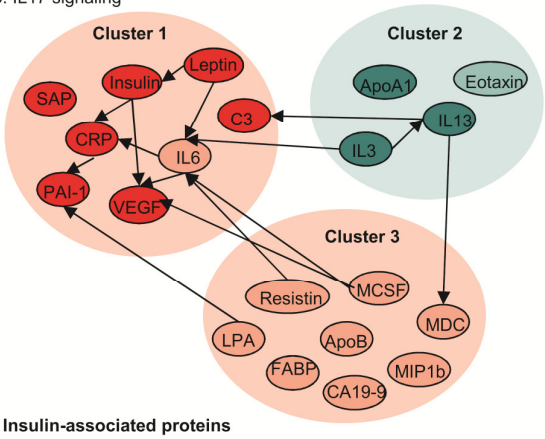
BMI-associated proteins

Pathways:

1. Acute phase response signaling
2. Glucocorticoid receptor signaling
3. Hepatic fibrosis
4. Role of cytokines in mediating communication between immune cells
5. IL17 signaling

Diseases:

1. Immunological disease
2. Inflammatory disease
3. Inflammatory response
4. Cardiovascular disease
5. Hematological disease



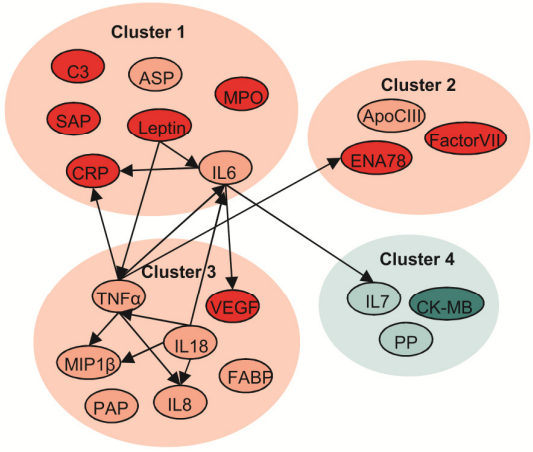
Insulin-associated proteins

Pathways:

1. Role of hypercytokinemia in the pathogenesis of Influenza
2. TREM1 signaling
3. Acute phase response signaling
4. Hepatic fibrosis
5. Role of cytokines in mediating communication between immune cells

Diseases:

1. Immunological disease
2. Inflammatory response
3. Renal and urological disease
4. Cardiovascular disease
5. Hematological disease



CHAPTER 5

Responses to high-fat challenges varying in fat type in subjects with different metabolic risk phenotypes

SJ van Dijk, M Mensink, D Esser, EJM Feskens, M Müller and LA Afman

ABSTRACT

Background: The ability of subjects to respond to nutritional challenges can reflect the robustness and flexibility of their biological system. Nutritional challenge tests could be used as an indicator of health status but more knowledge on metabolic and immune responses of different subjects to nutritional challenges is needed.
Objective: To compare the responses to high-fat challenges varying in fat type in subjects with different metabolic risk phenotypes.
Design: In a cross-over design 42 men consumed three high-fat shakes containing saturated fat (SFA), monounsaturated fat (MUFA) or n-3 polyunsaturated (PUFA). Men were selected on BMI and health status (lean healthy, obese healthy or obese diabetic) and phenotyped with MRI for adipose tissue distribution. Before and 2 and 4 hrs after shake consumption blood was drawn for measurement of plasma triglycerides (TAG), glucose, insulin, cytokines and expression of metabolic and inflammation-related genes in peripheral blood mononuclear cells (PBMCs) and ex vivo PBMC immune response capacity.
Results: Obese healthy and obese diabetic subjects had different metabolic and PBMC gene expression responses to high-fat challenges compared to lean healthy subjects. The MUFA challenge induced the most pronounced TAG response, mainly in obese healthy and obese diabetic subjects. The MUFA and n-3 PUFA challenge, compared to the SFA challenge, induced higher changes in expression of inflammation genes MCP1 and IL1β in PBMCs.
Conclusion: The metabolic response and PBMC gene expression response to high-fat challenges were affected by metabolic risk phenotype and fat type. Based on our results we suggest using a MUFA challenge to reveal differences in response capacity of subjects.

INTRODUCTION

In the western world food is generally continuously available and most of the day is spent in the postprandial state. Food intake can elicit a transient metabolic and low inflammatory response, especially when high fat is consumed [188-190]. The magnitude of this response reflects the ability of the biological system to adequately respond to nutrient intake. The presence of metabolic risk phenotypes such as obesity and type 2 diabetes might affect this ability as shown by elevated postprandial triglyceride levels in these subjects, in addition to metabolic abnormalities and chronic inflammation in the fasting state [191-195]. Not only the presence of overall obesity, but also body fat distribution, i.e. increased intra-abdominal (visceral) adipose tissue, might affect the metabolic condition and the postprandial triglyceride response [196].
Elevated postprandial triglyceride levels are considered as risk factors for CVD and were shown to be higher associated with cardiovascular events compared to fasting triglyceride levels [197, 198]. The response to nutrition might thus be considered as a better biomarker of health status than fasting measures, since this will reflect the metabolic robustness and metabolic flexibility of a person [199]. A widely applied example of a nutritional challenge test is the oral glucose tolerance test for measuring glucose clearance capacity. Nutritional challenge tests might be useful to detect small changes in health status, which could be of major importance in early detection and prevention of disease, but could also be used to test the effectiveness of (nutritional) interventions [199, 200]. However, at the moment relatively little is known about responses of different types of subjects to nutritional challenges such as a high-fat load which makes it difficult to use the response to challenges tests as biomarkers for the characterization of health status.
Postprandial meal studies showed that the type of fat consumed could affect the metabolic and inflammatory response [24, 189, 201-204] and gene expression responses in circulating peripheral blood mononuclear cells (PBMCs) [24]. PBMCs are immune cells that have been shown to be metabolically active [55], moreover, gene expression profiles of PBMCs were shown to reflect metabolic disease state and changes in nutrient intake which makes these cells an interesting target to investigate responses to nutritional challenges in different subjects [87, 205, 206].
The objective of this study is to characterize the metabolic, immune and PBMC response to high-fat challenges in subjects with different metabolic risk phenotypes. Responses to different types of fat (SFA, MUFA and n-3 PUFA) will be compared in order to reveal which type of fat may be best used in future high-fat challenges to test response capacity of subjects.

SUBJECTS AND METHODS

Subjects

In total 42 male Caucasian volunteers aged 50-70 years participated in the study. The study population consisted of three groups; 1) lean healthy (LH) subjects, 2) obese healthy (OH) subjects and 3) obese type 2 diabetic (OD) subjects. Subjects were excluded if they were vegetarian, regular tobacco smoker, allergic to dairy products or fish oil, current or recent user of fish oil supplements, ate more than four times fish/week, had an unstable body weight, used antibiotics or anti-inflammatory medication or had a long-term medical condition that could interfere with the study outcome. LH and OH subjects were excluded when using cholesterol-lowering medication; and lean subjects were excluded when using blood pressure lowering medication. OD subjects were all diagnosed with diabetes mellitus in the past, and did not use insulin and/or thiazolidinediones. During a screening visit an oral glucose tolerance test was performed and urinary glucose concentration was measured in healthy subjects to exclude the presence of (unknown) diabetes mellitus. Subjects were informed about the design and purpose of the study and all subjects provided written informed consent. The Medical Ethical Committee of Wageningen University (the Netherlands) approved the study.

Study design

All subjects randomly consumed three high-fat shakes enriched with either SFA, MUFA or n-3 PUFA in a crossover design, on three different days with at least one week between each study day.

The evening before the study day, subjects had to consume an identical low-fat meal and they were not allowed to eat or drink anything after 8 pm except water. The next morning, subjects came to the university and a fasting blood sample was collected. Blood was drawn into EDTA-containing tubes for plasma isolation and into BD Vacutainer Cell Preparation Tubes containing sodium citrate (Becton Dickinson, Breda, Netherlands) for PBMC isolation. After the first blood sample subjects was given a high-fat milkshake, which they had to consume within 15 min. Blood samples were collected 2 hours and 4 hours after consumption of the high-fat shake. During a study day, subjects were physically inactive and were asked not to eat or drink anything except water.

Shake composition

All high-fat shakes were isocaloric and differed only in fat composition. The shakes contained low-fat yoghurt, low-fat milk, strawberry flavour, 7.5 gr of sugar and 95 gr

of the test fat. The SFA shake contained 95 gr palm oil (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) and the MUFA shake contained 95 gr high-oleic acid sunflower oil (Aldoc BV, Schiedam, The Netherlands). The n-3 PUFA shake contained a combination of 40 gr palm oil and 55 gr Marinol D-40 (Lipid Nutrition, Wormerveer, The Netherlands) of which 40% was docosahexanoic acid (DHA). Vitamin E (165 mg Tocoblend L50, Vitablend, Wolvega, The Netherlands) was added to Marinol D-40 by the manufacturer to prevent oxidation. The same amount of vitamin E was added to the SFA and MUFA shakes. The macronutrient composition of the shakes was calculated based on the database of the Dutch Nutrient Databank and shown in table 1.

Table 1. Macronutrient composition of the shakes

	SFA shake	MUFA shake	n-3 PUFA shake
Energy (kJ)	4074	4074	4074
Energy (kcal)	987	987	987
Protein (g)	10	10	10
Carbohydrates (g)	22	22	22
Fat (g)	95	95	95
SFA (g)	51	8	32
MUFA (g)	37	79	25
PUFA (g)	6	8	38
EPA (g)	-	-	3
DHA (g)	-	-	23
Vitamin E (mg)	165	165	165

Abbreviations: Saturated fatty acids (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acid (PUFA), Eicosapentaenoic acid (EPA, C20:5), Docosahexaenoic acid (DHA, C22:6)

Body weight, body composition and abdominal fat distribution

Body weight was measured at screening and monitored during the study period. Body composition was determined on one study day by air-displacement plethysmography (BodPod; Life Measurement, Concord, CA) [85]. Abdominal fat distribution, i.e. abdominal visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT), was measured once during the study period in 17 LH, 18 OH and 4 OD subjects, using magnetic resonance imaging (MRI). Axial T1-weighted spin echo images were acquired with a Philips Gyroscan NT Intera 1.0T scanner using the body coil, with the subjects in supine position. A total of 14 10-mm-thick slices with no intersection gap were acquired, with the first slice at the superior border of the vertebral body of L5, and the remaining slices superiorly. Images were acquired during

breath-hold to avoid motion artefacts induced by breathing. Images were retrieved from the scanner using DICOM, and analysed using HIPPO (version 1.3), an IDL Virtual Machine 6.0-based freeware designed to quantify adipose tissue areas from MR images [207]. Automatically generated contour lines for SAT and VAT and the shape of Gaussian curve were manually adjusted by eye, as necessary, using the edit function. Retroperitoneal adipose tissue was excluded from VAT. VAT, SAT and VAT/SAT ratios derived from a single slice at the superior border of the vertebral body of L5 were used in the analysis.

Plasma glucose, insulin, triglycerides, free fatty acids

Immediately after blood was drawn in EDTA-containing tubes it was centrifuged (750 x g, 4°C, 10 min), and the plasma was stored at -80°C until analysis. Plasma FFA concentrations were analysed by the ACS-ACOD Method (NEFA HR kit, Wako Chemicals CmbH, Neuss, Germany). Plasma triglyceride and glucose concentrations were measured using the Dimension Clinical Chemistry System (Dade Behring Inc, USA). Glucose was measured by the Synchron LX20 System using hexokinase and glucose-6-phosphate dehydrogenase (Glucose reagent, Beckman Coulter, Fullerton, USA). Insulin concentrations were measured by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden).

Serum fatty acid composition in the triglyceride fraction was determined in pooled samples per group and per high-fat challenge at 0 hrs and 4 hrs and measured by gas-liquid chromatography as previously described [87].

Plasma cytokines

Plasma samples were analyzed on preformatted arrays (pro-inflammatory panel II, Meso Scale Diagnostics, LLC) on a SECTOR Imager 2400 reader (Meso Scale Diagnostics, LLC) for the measurement of IL1β and TNFα.

PBMC RNA isolation, cDNA synthesis and q-PCR

PBMCs were isolated from whole blood by using BD Vacutainer Cell Preparation Tubes according to the manufacturer's instructions. PBMC RNA was isolated by using the Qiagen RNeasy Micro kit (Qiagen, Venlo, Netherlands) and reverse transcribed using a cDNA synthesis kit (Promega, Leiden, Netherlands). Standard Q-PCR was performed using SensiMix real-time PCR reagents (Bioline, London, United Kingdom) and a Bio-Rad CFX384 machine (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). Primer sequences used were chosen based on the sequences available in PRIMERBANK (http://pga.mgh.harvard.edu/ primerbank/index.html). Q-PCR data were normalized by measuring cycle threshold ratios between candidate genes and a

housekeeping gene, human ribosomal protein LP0, which was shown to be consistent within PBMCs [208].
Baseline and postprandial gene expression changes of immune-related genes (*IL1b, IL8, MCP1, NFκB1, TNFα*) and lipid metabolism related genes (*ABCA1, PDK4, SREBP1, LDLr, LXRα, CYP27A1*) were determined. These genes were selected because we previously showed that their expression was affected by high-fat consumption [24].

Ex-vivo PBMC immune stimulation
PBMC immune response capacity in the fasted (0 hrs) condition and 4 hrs after the high-fat challenge was tested ex vivo and used as a measure of PBMC functionality. The ex vivo immune stimulation experiments were performed in a random subgroup of 13 LH and 15 OH subjects. Immediately after isolation PBMCs were re-suspended in RPMI 1640 culture medium with 10% heat-inactivated fetal calf serum and 1% penicillin and streptomycin. Cells in a concentration of 2.5 x 10^4 per ml were stimulated for 2 hours at 37 °C with 1 ng/ml lipopolysaccharide (LPS). Subsequently, cells were centrifuged and supernatants collected and stored at -80°C until analysis. TNFα produced by the PBMCs was measured in the supernatants by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems Europe Ltd, Abingdon, United Kingdom). LPS-stimulated TNFα production was corrected for TNFα production in non-stimulated cells.

Statistical analysis
The statistical package PASW (version 17.0; SPSS Inc. Chicago IL) and SAS (version 9.1, SAS Institute Inc. 2004, Cary, NC, USA) were used for analysis. Differences in baseline characteristics between the groups were analysed by analysis of variance (ANOVA) for single measures (i.e. age, body weight, body composition) or by linear mixed model for repeated measures (all other factors that were measured on all three consecutive study days). The analyses were followed by post-hoc LSD tests. Differences in responses for the different high-fat shakes and subject groups were analysed by linear mixed model. Delta values (changes from baseline) were used as dependent variables in the analysis and baseline values were included as covariables. Time, shake and group were used as fixed factors and time and shake as repeated factors in the model. When statistical significance was found, post-hoc LSD tests were performed to identify differences between shakes or groups.
The effect of VAT, SAT and VAT/SAT ratio on baseline and postprandial measures was only investigated within subject groups because of expected large differences in VAT and SAT between subject groups due to the predefined BMI categories. OD

subjects were excluded for this analysis due to the low number of subjects that was measured in the MRI scanner. For the analysis of the effect of body fat distribution VAT, SAT and VAT/SAT ratio were included as continuous covariables in the linear mixed model.

RESULTS

Subject baseline characteristics

Baseline subject characteristics are displayed in table 2. BMI, bodyfat percentage, abdominal VAT, abdominal SAT, plasma fasting TAG and insulin concentrations were significantly higher in the OH and OD subjects compared with the LH subjects. Fasting plasma glucose concentration was higher in the OD subjects compared with the LH and OH subjects.

Table 2 Baseline characteristics of the subjects in the three groups

	Lean Healthy (n=18)	Obese Healthy (n=18)	Obese Diabetic (n=6)
Age (yrs)	61.8 ± 5.9	62.6 ± 3.2	64.2 ± 4.6
BMI (kg/m^2)	23.8 ± 0.8	32.4 ± 3.0 *	33.5 ± 3.3 *
Body fat (%)	21.9 ± 5.7	38.2 ± 5.2 *	39.9 ± 5.1 *
VAT (cm^2) †	102 ± 40	231 ± 75 *	215 ± 31 *
SAT (cm^2) †	163 ± 52	350 ± 99 *	383 ± 42 *
VAT/SAT ratio†	0.64 ± 0.20	0.68 ± 0.24	0.57 ± 0.12
TAG (mmol/L)	1.5 ± 0.5	2.1 ± 0.9 *	2.0 ± 1.0 *
FFA (mmol/L)	0.51 ± 0.21	0.51 ± 0.13	0.58 ± 0.11
Insulin (mmol/L)	6.1 ± 2.8	13.4 ± 6.6 *	13.3 ± 7.8 *
Glucose (mmol/L)	5.2 ± 0.4	5.5 ± 0.4	7.2 ± 1.0 *

Data are presented as mean ± standard deviation. Abbreviations: Visceral adipose tissue (VAT), Subcutaneous Adipose Tissue (SAT), Free fatty acids (FFA), Triglycerides (TAG),

* Significantly different ($p<0.05$) from healthy lean subjects

† For VAT, SAT en VAT/SAT ratio n= 17 for lean healthy subjects, n=18 for obese healthy subjects and n=4 for obese diabetic subjects.

Plasma FFA, TAG, insulin and glucose responses

Changes in FFA, TAG, insulin and glucose concentration after the different high-fat challenges in the subject groups are depicted in table 3 and figure 1. Concentrations of FFA, TAG, insulin and glucose were changed after the high-fat challenges and the changes in all these metabolites were different between LH, OH and OD subjects. The changes in TAG, FFA, insulin and glucose concentrations were also depending on the type of fat consumed. A high MUFA challenge caused a higher total TAG response ($p<0.001$) and a reduced drop in FFA concentrations at 2 hours ($p<0.05$) compared with a SFA and n-3 PUFA challenge. The n-3 PUFA challenge caused a less pronounced increase in insulin concentrations ($p<0.01$) at 2 hrs compared with SFA and MUFA.

For TAG response an interaction effect between group and shake ($p=0.048$) was observed meaning that the postprandial TAG response was different for LH, OH and OD subjects depending on the type of high-fat shake consumed. No interaction between group and shake was found for FFA, insulin and glucose.

Table 3 Changes (mean ± sd) in metabolic parameters in plasma at 2 hrs and 4 hrs after high fat shake consumption

		SFA shake			MUFA shake			n-3 PUFA shake			Main effects			Interaction effects		
		0 hrs	Δ 2 hrs	Δ 4 hrs	0 hrs	Δ 2 hrs	Δ 4 hrs	0 hrs	Δ 2 hrs	Δ 4 hrs	group	time	shake	group* time	group* shake	shake* time
TAG	LH	1.53	0.64	0.48	1.37	0.91	1.41	1.50	0.33	0.78	0.889	<0.001	<0.001	0.002	0.048	<0.001
(mmol/L)		±0.49	±0.40	±0.48	±0.41	±0.47	±1.00	±0.50	±0.30	±0.58						
	OH	2.07	0.58	0.78	2.13	1.14	2.28	1.98	0.30	0.77						
		±0.99	±0.40	±0.61	±1.06	±0.68	±1.18	±0.78	±0.26	±0.45						
	OD	2.17	0.30	0.80	1.92	1.08	2.47	2.05	0.38	1.05						
		±1.16	±0.14	±1.21	±0.93	±0.49	±1.21	±1.21	±0.13	±0.49						
FFA	LH	0.51	-0.21	-0.04	0.49	-0.10	0.03	0.55	-0.25	-0.03	0.004	<0.001	0.002	0.016	0.390	0.002
(mmol/L)		±0.21	±0.19	±0.22	±0.20	±0.18	±0.23	±0.23	±0.21	±0.17						
	OH	0.48	-0.18	0.11	0.50	-0.09	0.08	0.54	-0.21	0.06						
		±0.10	±0.12	±0.11	±0.15	±0.16	±0.16	±0.15	±0.11	±0.16						
	OD	0.53	-0.20	0.07	0.58	-0.08	0.12	0.62	-0.12	0.09						
		±0.13	±0.12	±0.20	±0.13	±0.18	±0.28	±0.05	±0.25	±0.21						
Insulin	LH	6.52	3.08	-1.82	5.80	2.73	1.00	6.02	-0.02	-1.64	0.017	<0.001	<0.001	0.028	0.808	<0.001
(mmol/L)		±2.55	±3.77	±2.08	±3.38	±3.88	±3.98	±2.55	±3.77	±2.56						
	OH	13.52	6.59	-1.67	12.81	4.39	1.79	13.91	2.33	-2.04						
		±7.40	±9.60	±3.33	±5.24	±6.62	±6.07	±7.25	±5.87	±3.09						
	OD	12.66	8.85	0.03	13.57	7.43	2.66	13.79	1.27	-0.75						
		±7.50	±4.64	±1.40	±8.78	±3.71	±5.30	±8.49	±4.26	±4.20						
Glucose	LH	5.19	-0.30	-0.40	5.17	-0.29	-0.08	5.29	-0.42	-0.33	0.872	<0.001	0.002	<0.001	0.660	<0.001
(mmol/L)		±0.39	±0.35	±0.21	±0.35	±0.31	±0.28	±0.53	±0.39	±0.25						
	OH	5.50	-0.16	-0.43	5.53	-0.18	-0.31	5.54	-0.43	-0.52						
		±0.41	±0.48	±0.39	±0.43	±0.32	±0.28	±0.28	±0.38	±0.33						
	OD	7.22	0.47	-1.52	7.17	-0.03	-0.68	7.23	-0.48	-1.00						
		±0.94	±0.67	±0.48	±1.04	±1.14	±0.85	±1.25	±1.43	±0.68						

Abbreviations: Saturated fatty acids (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acid (PUFA), Triglycerides (TAG), Free fatty acids (FFA), Lean healthy (LH), Obese healthy (OH), Obese Diabetic (OD)

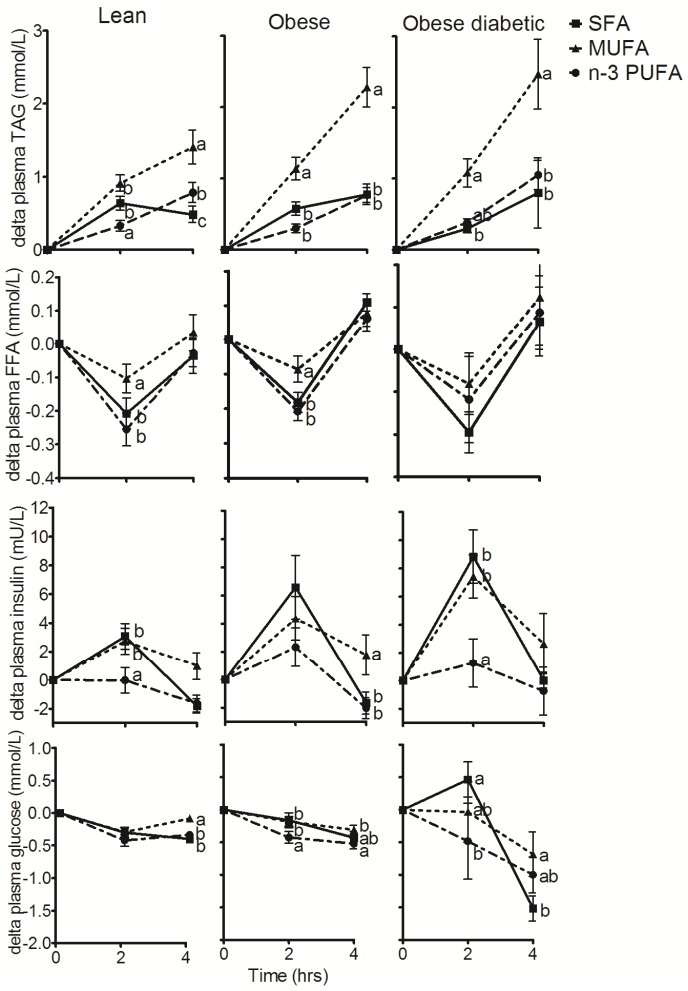


Figure 1 Mean (± SEM) effects of consumption of 3 shakes enriched in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (n-3 PUFA) on plasma TAG (A), FFA (B), Insulin (C) and Glucose (D). Different letters indicate significant differences ($p<0.05$) between shakes at a given time.

Serum fatty acid composition

Baseline values and changes in fatty acid composition of the TAG fraction in pooled serum samples 4 hours after the high-fat challenge are shown in table 4. The percentage palmitic acid was 1.1-fold higher after the SFA challenge, the percentage oleic acid was 1.6-fold higher after the MUFA challenge and the percentage DHA was 9.5-fold higher after the n-3 PUFA challenge, reflecting composition of the shakes.

Table 4 Serum fatty acid composition in the TAG fraction (% of total) of pooled samples for group and time point. Only values for palmitic acid, oleic acid and DHA are displayed

	SFA shake		MUFA shake		n-3 PUFA shake	
	0 hrs	Δ 4 hrs	0 hrs	Δ 4 hrs	0 hrs	Δ 4 hrs
Palmitic acid						
Lean healthy	27.45	2.87	25.76	-11.54	27.06	-0.56
Obese healthy	28.14	2.54	27.89	-11.22	29.13	0.30
Obese diabetic	27.77	3.74	26.99	-11.09	28.74	0.32
Oleic acid						
Lean healthy	35.78	2.52	38.14	22.79	37.41	-2.48
Obese healthy	36.14	1.12	36.02	20.68	35.71	-0.55
Obese diabetic	36.79	0.72	39.02	20.21	36.73	-1.48
DHA						
Lean healthy	0.68	-0.09	0.44	-0.12	0.32	4.56
Obese healthy	0.66	-0.09	0.63	-0.16	0.55	2.86
Obese diabetic	0.96	0.27	0.90	-0.39	0.54	3.31

PBMC gene expression

Gene expression level at baseline (fasting) was not different between the subject groups. The high-fat challenge altered expression of several metabolic genes and inflammation-related genes (table 5). Changes in expression of the metabolic genes *ABCA1* and *LDLr* and the inflammation-related genes *IL1b* and *MCP1* were also depending on whether the subjects were LH, OH or OD. Changes in expression of *ABCA1* and *LDLr* were less pronounced for OH and OD subjects ($p<0.05$) compared with LH subjects. Differences in expression changes of *IL1b* and *MCP1* were most likely due to higher increases of expression of these genes in OD subjects compared with LH and OH subjects, since the effects were not significant when OD subjects were excluded from analysis.

Changes in expression of *PDK4, LDLr, IL8* and *MCP1* expression were depending on the type of fat in the challenge. When OD subjects were excluded from analysis also *IL1b* expression was different between the fat challenges. The MUFA challenge induced a lower decrease in *PDK4* expression at 2 hours ($p<0.001$) compared with the other challenges. The MUFA and n-3 PUFA challenge induced a higher change in *MCP1* and *IL8* expression at 4 hrs ($p<0.05$) compared with the SFA challenge. There were no interaction effects between subject groups and shakes for the measured genes.

PBMC immune response capacity

Fasting levels and changes in PBMC immune response capacity after the high-fat challenges, measured as ex vivo LPS-stimulated TNFα production of PBMCs, were neither depending on the metabolic risk profile of the subjects, nor depending on the type of fat in the challenge (table 6). However, TNFα production was significantly lower ($P<0.001$) 4hrs after the high-fat challenge compared with the fasting state.

Plasma cytokines

Fasting plasma IL1β concentration was higher in the OD subjects compared to LH and OH subjects (table 6). Plasma concentrations of TNFα after the challenge were also different between the groups, with lower TNFα concentrations in LH compared with OH and OD subjects ($p<0.05$), however TNFα responses were not different between the groups. Changes in plasma concentrations of IL1β varied according to the type of fat in the challenge and whether the subjects were LH, OH or OD. These group and shake effects were probably mainly due to the different IL1β responses to the different types of fat in OD subjects, since the shake and shake* group effects were not significant when the OD subjects were excluded from the analysis.

Abdominal fat distribution

Within the group of LH subjects and within the group of OH subjects there was no significant influence of abdominal fat distribution (i.e. VAT, SAT or VAT/SAT) and changes in plasma FFA, TAG, insulin or glucose concentrations, changes in PBMC gene expression, or changes in cytokine levels in response to the high-fat challenges.

Table 5 Changes (mean ± sd) in PBMC gene expression at 2 hrs and 4 hrs after high-fat shake

		SFA shake		MUFA shake		n-3 PUFA shake		Main effects			Interaction effects		
		Δ 2 hrs	Δ 4 hrs	Δ 2 hrs	Δ 4 hrs	Δ 2 hrs	Δ 4 hrs	group	time	shake	group* time	group* shake	shake* time
Metabolism													
ABCA1	LH	0.49± 1.03	1.05± 0.78	0.36± 0.62	0.59± 0.70	0.42± 0.89	0.39± 0.70	0.007	<0.001	0.235	0.038	0.877	0.261
	OH	0.42± 0.84	0.28± 0.93	0.42± 0.84	0.36± 1.11	0.03± 0.55	0.03± 0.62						
	OD	-0.11±0.75	0.34± 0.77	0.31± 0.70	0.10± 0.75	0.36± 0.97	0.41± 1.04						
CYP27A1	LH	0.41± 0.60	0.53± 0.55	0.41± 0.63	0.66± 0.49	0.39± 0.43	0.65± 0.65	0.576	<0.001	0.682	0.325	0.704	0.921
	OH	0.40± 0.53	0.34± 0.64	0.47± 0.72	0.60± 1.07	0.25± 0.63	0.32± 0.58						
	OD	0.10± 0.53	0.44± 0.75	0.14± 0.50	0.21± 0.55	0.53± 0.44	0.50± 0.46						
LDLr	LH	-0.45±0.49	-0.40±0.58	-0.67±0.59	-0.19±0.57	-0.51±0.46	-0.42±0.41	0.034*	<0.001	0.040*	<0.001	0.642	0.025
	OH	-0.28±0.46	-0.16±0.52	-0.28±0.58	0.03± 0.80	-0.31±0.48	-0.24±0.59						
	OD	-0.16±0.33	0.01± 0.56	-0.11±0.45	0.33± 0.40	0.17± 0.46	0.33±0.49						
LXRα	LH	0.06± 0.61	0.21± 0.73	0.04± 0.73	0.05± 0.66	-0.02±0.48	-0.16±0.59	0.225	0.789	0.904	0.620	0.937	0.108
	OH	-0.05±0.74	0.12± 0.72	-0.23±0.81	-0.25±0.81	0.05± 0.39	-0.02±0.40						
	OD	-0.25±0.38	0.20± 0.74	0.26± 0.33	0.10± 0.55	0.48± 0.86	0.13± 0.87						
PDK4	LH	-0.28±0.64	0.39± 0.69	0.00± 0.53	0.53± 0.60	-0.55±0.43	0.46± 0.60	0.563	<0.001	<0.001	0.292	0.770	<0.001
	OH	-0.28±0.61	0.25± 0.65	0.06± 0.37	0.55± 0.46	-0.47±0.31	0.29± 0.64						
	OD	-0.22±0.50	0.42± 0.64	0.58± 1.55	0.88± 1.63	-0.38±0.50	0.28± 0.39						
SREBP1	LH	-0.05±0.54	0.13±0.38	0.16±0.53	0.32±0.51	0.17±0.48	0.05±0.69	0.006*	0.343	0.879	0.338	0.638	0.379
	OH	-0.08±0.62	-0.06±0.62	0.16±0.81	0.12±0.76	0.06±0.31	-0.03±0.43						
	OD	-0.11±0.37	0.02±0.63	-0.30±0.58	0.04±0.84	-0.06±0.59	0.12±0.53						

		SFA shake		MUFA shake		n-3 PUFA shake		Main effects			Interaction effects		
		Δ 2 hrs	Δ 4 hrs	Δ 2 hrs	Δ 4 hrs	Δ 2 hrs	Δ 4 hrs	group	time	shake	group* time	group* shake	shake* time
Inflammation													
IL1b	LH	0.13± 0.64	0.02± 0.62	0.07± 0.78	0.41± 0.87	-0.08±0.37	0.07± 0.85	0.351	<0.001*	0.293†	0.014*	0.638	0.167
	OH	-0.13±1.16	-0.11±0.65	0.08± 0.71	0.18± 1.10	-0.11±0.60	0.23± 0.56						
	OD	0.35± 0.49	0.54± 0.85	0.61± 1.73	1.17± 1.99	0.18± 0.72	0.79± 1.04						
IL8	LH	0.20± 1.46	0.98± 1.28	0.10± 1.16	1.89± 1.35	-0.35±1.15	1.68± 1.83	0.359	<0.001	0.032	0.239	0.932	0.003
	OH	-0.86±2.09	0.68± 1.38	0.18± 1.44	1.59± 1.67	-0.66±1.18	2.01± 1.36						
	OD	0.02± 1.00	1.74± 0.70	0.27± 1.42	2.04± 1.29	0.13± 2.68	2.48± 1.84						
MCP1	LH	0.48± 1.11	0.31± 0.91	0.37± 1.49	1.37± 1.51	0.39± 0.92	0.94± 1.36	0.500	<0.001	0.006	0.037*	0.730	0.015
	OH	-0.02±1.05	0.22± 1.40	0.36± 1.51	0.97± 1.89	0.29± 0.88	0.77± 0.69						
	OD	0.65± 1.22	1.11± 1.08	1.22± 0.69	1.96± 0.93	0.75± 1.55	1.77± 1.67						
NFκB1	LH	0.09±0.78	0.21± 0.51	-0.13±0.46	-0.16±0.85	0.07±0.47	-0.07±0.56	0.113	0.927	0.870	0.808	0.278	0.877
	OH	-0.04±0.61	-0.28±0.59	0.02±0.72	-0.16±0.87	-0.03±0.37	0.10±0.69						
	OD	0.03±0.39	0.08±0.47	0.14±0.50	0.10± 0.58	-0.05±0.99	0.08±0.64						
TNFa	LH	0.40± 0.55	0.33± 0.44	0.45± 0.65	0.27± 0.77	0.26± 0.42	0.13± 0.47	0.761	<0.001	0.085	0.280	0.750	0.529
	OH	0.19± 0.57	0.06± 0.40	0.38± 0.69	0.25± 0.84	0.33± 0.50	0.37± 0.55						
	OD	0.51± 0.52	0.61± 0.45	0.48± 0.49	0.57± 0.50	-0.09±0.55	0.09± 0.51						

*no significant effect when OD subjects are excluded from analysis, †significant effect (p 0.034) when OD subjects are excluded from analysis

Abbreviations: Saturated fatty acids (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acid (PUFA), Lean healthy (LH), Obese healthy (OH), Obese Diabetic (OD), ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1), low density lipoprotein receptor (LDLr), liver X receptor (LXR), pyruvate dehydrogenase kinase, isozyme 4 (PDK4), sterol regulatory element binding transcription factor 1 (SREBP1), Monocyte chemotactic protein 1 (MCP1), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFκB1), tumor necrosis factor alpha (TNFα)

Table 6 Changes (mean ± sd) in plasma cytokines and ex vivo LPS-stimulated TNFα production of PBMCs after high fat shake consumption

		SFA shake			MUFA shake			n-3 PUFA shake			Main effects			Interaction effects		
		0 hrs	Δ 2 hrs	Δ 4 hrs	0 hrs	Δ 2 hrs	Δ 4 hrs	0 hrs	Δ 2 hrs	Δ 4 hrs	group	time	shake	group* time	group* shake	shake* time
Plasma																
IL1β	LH	0.75	0.02	0.00	0.68	0.05	0.00	0.69	0.02	0.02	0.512	0.740	0.036*	0.676	0.041*	0.784
(pg/mL)		±0.42	±0.19	±0.16	±0.30	±0.10	±0.16	±0.27	±0.15	±0.16						
	OH	0.70	0.09	0.07	0.75	0.00	0.04	0.76	0.05	-0.01						
		±0.51	±0.14	±0.12	±0.49	±0.10	±0.16	±0.55	±0.25	±0.10						
	OD[†]	1.44	0.01	-0.06	1.29	0.12	0.17	1.60	-0.24	-0.07						
		±1.03	±0.12	±0.32	±0.97	±0.48	±0.34	±0.93	±0.45	±0.58						
TNFα	LH	6.54	0.04	-0.14	6.43	-0.19	-0.20	6.73	-0.26	-0.11	0.004	0.280	0.993	0.369	0.536	0.295
(pg/mL)		±1.55	±0.54	±0.64	±1.48	±0.64	±0.54	±1.76	±0.60	±1.00						
	OH	7.27	0.22	-0.02	7.31	0.14	0.07	7.58	-0.10	-0.09						
		±2.04	±0.53	±0.60	±1.68	±0.55	±0.67	±1.81	±0.65	±0.86						
	OD	7.93	0.14	-0.28	8.15	0.19	-0.17	7.57	0.21	0.29						
		±1.35	±0.33	±0.52	±1.50	±0.78	±0.41	±1.54	±0.66	±1.11						
Ex vivo LPS-stimulated[#]																
TNFα	LH	164		-26	138		-5	210		-20	0.063	<0.001	0.900	0.084	0.962	0.639
(pg/mL)		±92		±72	±95		±85	±262		±64						
	OH	141		-50	162		-50	170		-67						
		±86		±86	±130		±137	±113		±84						

* no significant effect when OD subjects are excluded from analysis, [†] baseline values significantly different from those of LH and OH subjects, [#] This measurement was done in a subsample of 13 LH and 15 OH subjects.

Abbreviations: Lean healthy (LH), Obese healthy (OH), Obese Diabetic (OD)

DISCUSSION

Within this comprehensive study we showed that responses to high fat challenges in subjects with different metabolic risk phenotypes were characterized by differences in the plasma metabolic parameters TAG, FFA, glucose and insulin and in expression of the metabolic genes *ABCA1, LDLr* and inflammatory genes *IL1β* and *MCP1* in PBMCs. Moreover, comparison of responses to high-fat challenges high in SFA, MUFA or n-3 PUFA showed that a high MUFA challenge induced the most pronounced response, especially for TAG.

The high-fat challenge-induced differential changes in TAG and insulin concentrations in LH, OH and OD subjects are in line with results from other postprandial studies showing higher TAG and insulin responses in obese and diabetic subjects after high-fat meal consumption [191, 194, 195, 209, 210]. We consider the differential changes in TAG and insulin as a reflection of reduced cellular adaptation capacity to respond to a high-fat challenge in the presence of obesity and/or diabetes.

The change in expression of the cholesterol metabolism genes *ABCA1* and *LDLr* in lean subjects in response to the challenge was comparable with observations in lean subjects in earlier studies performed by us and others [24, 211]. The new finding of less pronounced changes in expression of these genes in OH and OD subjects may reflect a less optimal metabolic adaptation response of their PBMCs to a high-fat challenge. This might be caused by a lower lipid uptake by the cells and/or a lower activation of gene expression in response to high-fat intake, possibly due to lowered sensitivity of the cells to lipids. Changes in expression of *ABCA1* and *LDLr* in response to a high-fat challenge might thus be considered as potential markers of health status.

Expression changes of the inflammatory genes *IL1β* and *MCP1* in response to high-fat challenges were also different between subjects groups, but since these effects were mainly due to different changes in OD subjects, and not in OH subjects, we might consider changes in expression of these genes as indicators of a more severe metabolic phenotype. Higher changes in IL1β expression were not accompanied by higher changes in plasma IL1β concentrations. One of the reasons for this difference could be the fact that IL1β is also produced by other cells than PBMCs. A potential other explanation for the absence of differences in plasma cytokines is the low power to detect significant effects in the small OD subject group with high variation in cytokine response.

The amount of VAT, SAT or VAT/SAT did not influence the response to a high-fat challenge within subject groups. Although the number of subjects in each subgroup might have been too small to detect a significant effect of fat distribution, it is also arguable whether high VAT is strongly affecting metabolic health; some studies found

effects of VAT, mainly on TAG response [196, 212, 213], but others [214, 215] suggested that liver fat might be a more sensitive determinant of metabolic health.
The plasma metabolic and PBMC gene expression response to the challenge was also depending on the main type of fat present. The MUFA challenge had a stronger TAG-raising effect compared with the SFA and n-3 PUFA challenges. This effect was more pronounced in OH and OD subjects, suggesting that high MUFA intake might be a stronger metabolic challenge for subjects with a metabolic risk phenotype. In our study the MUFA challenge revealed differences in TAG response between lean and obese subjects that were both defined as healthy. It should be further investigated in other subject groups whether the plasma TAG response to a MUFA challenge could be used as a biomarker to detect small differences in health status in subjects with more comparable phenotypes.
The MUFA challenge may have been the most challenging for the biological system because it contained almost exclusively oleic acid (83% of total fat) while the other shakes contained a mixture of fatty acids. The body might have difficulties metabolizing this high dose of a single fatty acid as mixtures of fatty acids are more commonly consumed. Habitual intake of fish n-3 PUFA is low and therefore we prepared a n-3 PUFA shake consisting of a mixture of palm oil and 55 gr fish oil, a well-tolerated dose in a former study at our group [24]. In the latter study, consumption of a n-3 PUFA shake containing this high dose of 55 gr n-3 PUFA and no palm oil, decreased the expression of LXR signalling genes in PBMCs of young, lean men when compared to consumption of a SFA shake with 55 gr butter. Although we gave a higher total fat load in our study, we observed no expression differences for these genes. This may be due to differences in fat types (palm oil vs. butter), shake composition, sampling time (4 hrs vs 6 hrs) or age of the subjects.
Remarkably, the n-3 PUFA shake in our study induced a lower increase in insulin concentration compared with the other shakes. Only few other studies have reported acute insulin-lowering effects of n-3 PUFA intake [201, 216]. Peak insulin concentrations are reached normally between 0-2 hours after a meal [191, 204, 217], but we lack data for these early time points. n-3 PUFA intake might have caused an earlier insulin peak resulting in lower insulin concentrations at later time points.
The n-3 PUFA and the MUFA challenge induced higher changes in *MCP1* and *IL8* expression compared to the SFA challenge, which is in line with findings from a previous study showing that acute high n-3 PUFA intake induced a pro-inflammatory PBMCs gene expression profile [24]. A possible explanation for this induction is that unsaturated fatty acids are more prone to oxidation than SFA and might induce more oxidative stress and in turn affect inflammatory status. Another explanation may be that palmitic acid, because it is in general more regularly consumed, poses less stress to

PBMCs resulting in lower expression changes of inflammation genes than high doses of oleic acid or DHA. A high-fat shake consisting of palm oil may thus be less suitable to challenge the system compared to shakes containing unsaturated fats.
All high-fat challenges reduced PBMC immune response capacity but whether this reduction is an indication of lower inflammatory status or diminished immune functioning of the cells cannot be distinguished. As only effects after a challenge were observed an effect of circadian rhythm on PBMCs cannot be excluded. Since differences in PBMC immune response capacity between subject groups did not reach significance, this ex vivo challenge might not be a sensitive indicator for differences in health status.
In our study we monitored different aspects of the response to high-fat challenges i.e. plasma metabolites, inflammatory proteins, PBMC gene expression and immune functioning. We selected these determinants based on literature and previous transcriptome profiling [24]. We were able to detect differential changes in expression of metabolic parameters and certain genes between the selected subject groups differing in BMI and health status. However, for the identification of small differences between responses of subjects that are phenotypically more similar, more sensitive monitoring of the challenge response is needed. Whole genome transcriptome, proteome or metabolome profiling tools could be used for a more extensive characterization of the challenge response. As shown in previous studies, extensive profiling could identify subtle changes of genes, proteins or metabolites in pathways and clusters [200, 218]. The combination of challenge tests and extensive profiling may result in detection of changes in health status at a very early stage [62].
In conclusion, of the three fat types studied most pronounced changes were seen for the high oleic sunflower oil (MUFA), this fat type seems to be the most promising to be applied in challenge studies to test metabolic response capacity. Moreover, we identified a combination of plasma metabolic measures and genes expressed in PBMCs that were differently responding to a high-fat challenge in subjects with distinct metabolic risk phenotypes. These potential markers are likely candidates to be further tested and used in high-fat challenge tests to define metabolic response capacity of subjects.

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CHAPTER 6

Changes in PBMC gene expression profiles of healthy subjects and subjects with the metabolic syndrome in response to a challenge of extreme caloric restriction

SJ van Dijk, A Michalsen, M Müller and LA Afman

ABSTRACT

Background: Extreme caloric restriction (CR) is applied for rapid weight loss and other health reasons but presents a challenge to the human body. Subjects with metabolic syndrome (MetS) show several abnormalities in their metabolic system and this might affect their ability to deal with a period of extreme CR .
Objective: To identify whether basal PBMC gene expression profiles are different between MetS subjects and healthy subjects and whether these subjects will respond differently to a challenge of extreme CR.
Design: Nine women with MetS (≥3 MetS characteristics) and ten healthy women (≤ 1 MetS characteristics) received a run-in low-caloric diet for 2 days followed by an extreme CR period of 7 days (250 kcal/day). Blood was drawn after run-in (day 0) and at after 3 and 7 days of CR. PBMCs were isolated and samples from day 0 and 7 were used for whole genome expression analysis.
Results: Before CR 809 genes were differently expressed between MetS subjects and healthy controls, while after CR this increased to 1394 differently expressed genes. Major differences between MetS subjects and controls were seen for genes involved in mitochondrial energy metabolism and these differences became more pronounced after CR. The subject groups showed differences in response to CR. Healthy subjects showed a downregulation of immune-related genes, while this was not clearly seen in MetS subjects.
Conclusion: A metabolic challenge in the form of CR enlarged differences in PBMC gene expression between MetS subjects and healthy controls. The differences in PBMC gene expression response to fasting suggests that differences in metabolic status can play an important role in adaptive molecular responses to metabolic challenges.

INTRODUCTION

The prevalence of obesity has become alarmingly high in the last decade which poses a serious threat to public health. Obesity has been associated with other metabolic risk factors including glucose intolerance, hypertension and dyslipidemia, which often cluster together, collectively referred to as the metabolic syndrome. The current increase in obesity and metabolic syndrome can be largely attributed to changes in diet. In the past our ancestors faced alternate periods of fasting and overfeeding, depending on the availability of food. In order to survive periods of food shortage the human body has evolved to regulate efficient energy storage during periods of overfeeding. However, nowadays there is continuous nutritional abundance and this in combination with a lower need for physical activity has led to the obesity epidemic.

Periods of voluntary fasting are still practiced worldwide, mostly for religious reasons, but extreme caloric restriction (CR) is also common practice among overweight people desiring rapid weight loss [219]. Moreover, fasting has found its way in health retreats where it is considered as a way to 'clean' or detoxify the body and thereby improve health. Also in the field of integrative medicine fasting is used as a method to enhance lifestyle modification [220].

Long term mild CR has been demonstrated to increase longevity in several animal models and has beneficial health effects in humans as well [221, 222]. However, although mild long-term CR has been proven to be beneficial for human health, scientific evidence for health effects of short-term extreme CR regimes in which only water or juice is consumed is limited. This extreme type of CR will not only lead to rapid weight loss but will also stress the human body requiring adequate metabolic adaptations to maintain homeostasis. During CR the body needs to slow down metabolic rate and has to shift from the use of carbohydrate as an energy source to fat and protein in order to conserve glucose. It has been shown that subjects with characteristics of the metabolic syndrome, compared to healthy subjects, experience difficulties with handling of metabolic stressful challenges such as high lipid or glucose loads [223, 224]. Moreover, the presence of metabolic syndrome is associated with metabolic disturbances, such as elevated lipid and glucose levels, and low-grade inflammation in the fasting state [225]. Previous studies have demonstrated that differences between healthy subject and obese or diabetic subjects can be reflected in PBMC gene expression [134, 226]. Moreover, gene regulation of PBMCs can be affected by challenges such as fasting [87].

In this study we will investigate whether one week of extreme CR has different effects on PBMC whole genome expression in metabolic syndrome subjects compared to

healthy subjects and whether the CR challenge affects the potential differences in PBMC gene expression of both groups of subjects.

SUBJECTS AND METHODS

Subjects

In total, 19 female subjects who visited the Kliniken Essen-Mitte (Essen, Germany) were willing to participate in the study. The healthy control group (n=10) consisted of subjects with ≤ 1 out of the 5 NIH criteria for MetS (i.e. abdominal obesity waist circumference >88 cm, blood pressure > 130/85 mm Hg, fasting plasma glucose >5.9 mmol/L, HDL <1.3 mmol/L, TAG>1.7 mmol/L). The MetS group (n=9) consisted of subjects with ≥3 criteria for MetS.

Total serum cholesterol, low-density and high-density cholesterol, and triglycerides were measured in a Synchron LX 20 (Beckman, Brea, CA, USA). Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. Serum fasting insulin was determined by microparticle enzyme immunoassay (Insulin RIA DSL-1600, Diagnostic Systems Laboratories, Sinsheim, Germany). Plasma free fatty acids concentrations were analysed by the ACS-ACOD Method (NEFA HR kit, Wako Chemicals CmbH, Neuss, Germany).

Study design

All subjects received a run-in low-caloric diet (800-1000 kcal/day) for 2 days before the start of the extreme CR period. This run-in diet contained fruit, vegetables and pure rice or potatoes. Subjects subsequently followed a 7-day CR regime which started the evening of run-in. During this CR period the daily energy intake was 250 kcal/day consisting of 250-300 ml of fruit juice (apple juice, mixed vegetable juice, carrot juice or orange juice, as per choice).

PBMC and RNA isolation

PBMCs were isolated from whole blood on day 0, 3 and 7 using Optiprep (Axis-shield, Oslo, Norway) according to the manufacturer's manual. Subsequent RNA isolation was performed using the Qiagen RNAeasy kit (Qiagen, Venlo, the Netherlands).

The RNA yield was quantified with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and RNA integrity was checked on an Agilent 2100 bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom). Only samples with a RIN value >8 were selected for microarray analysis.

Microarray processing

Total RNA (500 ng/sample) from the PBMC samples at day 0 and day 7 was labeled by using an one-cycle cDNA labeling kit (MessageAmpTM II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk a/d IJssel, Netherlands) and hybridized to human whole-genome GeneChip arrays encoding 17,699 genes, designed by the European Nutrigenomics Organization (NuGO) and manufactured by Affymetrix (Santa Clara, CA). Sample labeling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

Microarray data analysis

Quality control was performed and fulfilled the criteria for array hybridization suggested by the Tumor Analysis Best Practices Working Group [91]. Microarrays were analyzed using the reorganized oligonucleotide probes as described by Dai et al. [92]. They combined all individual probes for a gene, enabling the possibility to detect overall transcription activity, based on the latest genome and transcriptome information, instead of the Affymetrix probe set annotation**.** Expression values were calculated with the Robust Multichip Average (RMA) method and normalization was done using quantile normalization [93, 94].

Only probe sets with normalized signals above 20 on at least 5 arrays were defined as expressed and selected for analysis.

Genes were defined as different between the subject groups when comparison of the average normalized signal intensities showed a p-value below 0.05 in a t-test with Bayesian correction (Limma) [95]. Genes were defined as changed by CR in each subject group when comparison of the average normalized signal intensities showed a p-value below 0.05 in a paired t-test with Bayesian correction (Limma). Genes were defined as differently changed between the subject groups when comparison of the average changes in normalized signal intensities showed a p-value below 0.05 in a t-test with Bayesian correction (Limma).

Data were further analyzed with the use of Ingenuity Pathway Analysis (Ingenuity Systems). This analysis identified canonical pathways that were most significant to the data.

Statistical analyses

The statistical package PASW (version 18.0; SPSS Inc, Chicago,IL) was used for analysis of the data. Differences between subject groups were determined by unpaired t-tests. Changes within subjects groups were determined by paired t tests.

RESULTS

Subject characteristics

Baseline characteristics of the healthy control and MetS subjects are summarized in table 1.

Body weight decreased after CR in the control subjects (-5.6 ± 1.2 kg) and in the MetS subjects (-5.8 ±2.1 kg), but weight changes were not significantly different between groups.

Table 1 Subject baseline characteristics (mean ± st dev)

	Healthy subjects (n=10)	MetS subjects (n=9)
Age (yrs)	44.0 ± 8.0	52.9 ± 7.1[a]
BMI (kg/m2)	25.4 ± 3.5	36.3 ± 6.9[a]
Weight (kg)	72.1 ± 10.1	103.5± 17.3[a]
Total cholesterol (mmol/L)	5.23 ± 0.77	5.51 ± 1.37
LDL cholesterol (mmol/L)	3.03 ± 0.68	3.76 ± 1.26
HDL cholesterol (mmol/L)	1.77 ± 0.33	0.99 ± 0.27[a]
TAG (mmol/L)	0.79 ± 0.34	1.71 ± 0.59[a]
Insulin (mmol/L)	10.8 ± 12.3	16.9 ± 17.2
Glucose (mmol/L)	4.8 ± 0.6	5.5 ± 0.7[a]
FFA (mmol/L)	0.42 ± 0.18	0.45 ± 0.20
MetS characteristics	0: n=5 1: n=5	3: n=3 4: n=2 5: n=4

[a] significantly different ($p<0.05$) from healthy controls

Microarray analyzes

Differences between PBMC gene expression profiles of MetS and control subjects

Before CR 809 genes were differently expressed between MetS and control subjects and this number increased to 1394 differently expressed genes after CR, 393 of these genes were differently expressed between MetS and control subjects both before and after CR (figure 1A). Most differentially expressed genes (63%) were lower expressed in MetS compared with controls and this became more pronounced after CR (figure 1C).

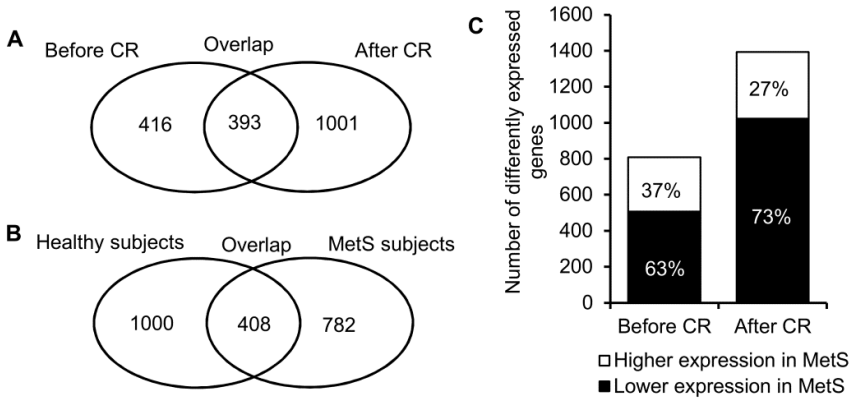


Figure 1 Number of genes changed for each comparison analysis. A: number of genes differently expressed between healthy subjects and MetS subjects, both before and after caloric restriction (CR). B: number of genes changed by CR in healthy subjects and MetS subjects. C: number and percentage of differently higher and lower expressed genes in PBMCs between MetS subjects and healthy control subjects before and after CR.

Ingenuity pathway analyzes revealed that several significantly lower expressed genes in MetS subjects both before and after CR were involved in pathways related to energy metabolism (i.e. oxidative phosphorylation (OXPHOS), mitochondrial dysfunction, ubiquinone biosynthesis), estrogen receptor signaling and pyrimidine metabolism (figure 2). The differences between MetS subjects and controls in most of these pathways became more pronounced after CR.

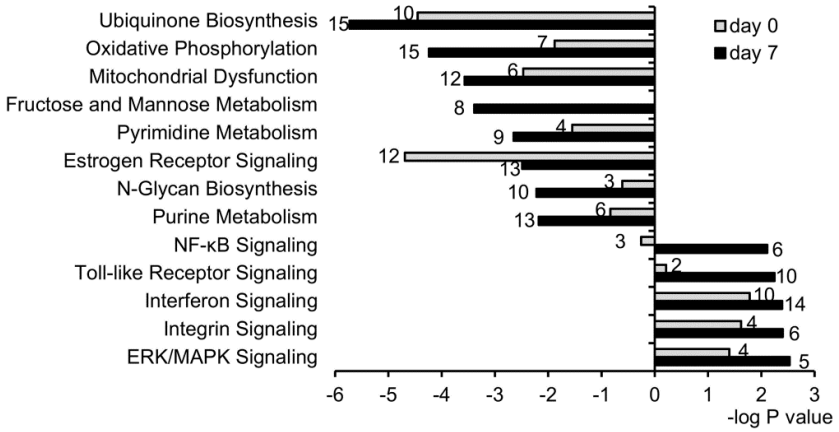


Figure 2 Significantly different pathways between MetS and controls before (day 0, grey bar) and after (day 7, black bar) caloric restriction (CR). Only pathways with a p value <0.01 before and/or after CR were selected. A negative –log p value indicates that the pathway is lower expressed in MetS subjects compared with control subjects and a positive –log p value indicates that the pathway is higher expressed in MetS subjects compared with control subjects

Figure 3 shows that the expression levels of genes involved in these pathways decreased with the presence of a higher number of MetS characteristics. In addition, several genes encoding mitochondrial ribosomal proteins (*MRPs*) and ribosomal proteins were lower expressed in MetS subjects, and the number of differently expressed (mitochondrial) ribosomal proteins was increased after CR (figure 3 and supplemental table 1) and expression levels are decreased with the presence of a higher number of MetS characteristics. Gene expression of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PGC1α*), a regulator of mitochondrial energy production, and expression of dihydroorotate dehydrogenase (*DHODH*), involved in pyrimidine biosynthesis, was also lower in MetS subjects (supplemental table 1).
Pathway analysis revealed that significantly higher expressed genes in MetS subjects were involved in several signaling pathways related to the immune response such as Toll-like receptor signaling and NFκB signaling. Differences between subject groups in most of these pathways became more pronounced after CR.
Further analysis showed that several genes involved in cell adhesion and platelet activation e.g. *vWF, GP1AB, GP1BB, GP6, GP9, ITGA2B, ITGB3, SELP* were higher expressed in MetS subjects, mainly before CR (figure 3 and supplemental table 1).

Differences in response to CR of MetS and healthy control subjects

CR changed the expression of 1408 genes in healthy control subjects and of 1190 genes in MetS subjects, of these genes the expression 408 genes was changed in both subject groups (figure 1B). Further analysis showed that the expression of 303 genes was differently changed by CR between MetS and control subjects. Ingenuity pathway analysis revealed that genes related to immune processes were significantly differently changed between the subjects groups (table 2). Of these genes several were involved in natural killer (NK)-cell response and were downregulated in controls and not in MetS subjects. Among these genes were NK-cell receptors of the C-type lectin family such as i.e. *KLRB1, KLRD1, KLRF1, KLRG1* and also genes involved in the NK-cell mediated release of cytolytic granules i.e. perforin, granzymes, granulysin. In addition, expression of chemokines *CCL4* and *CCL5*, involved in activation of immune cells, was reduced in healthy controls and not in MetS subjects. Also genes involved in platelet activation were downregulated in control subjects but not in MetS subjects, as shown in figure 4. A few other immune-related genes such as *EREG, PTGS2* and *EGR1* were specifically upregulated in the MetS subjects and not in the controls.

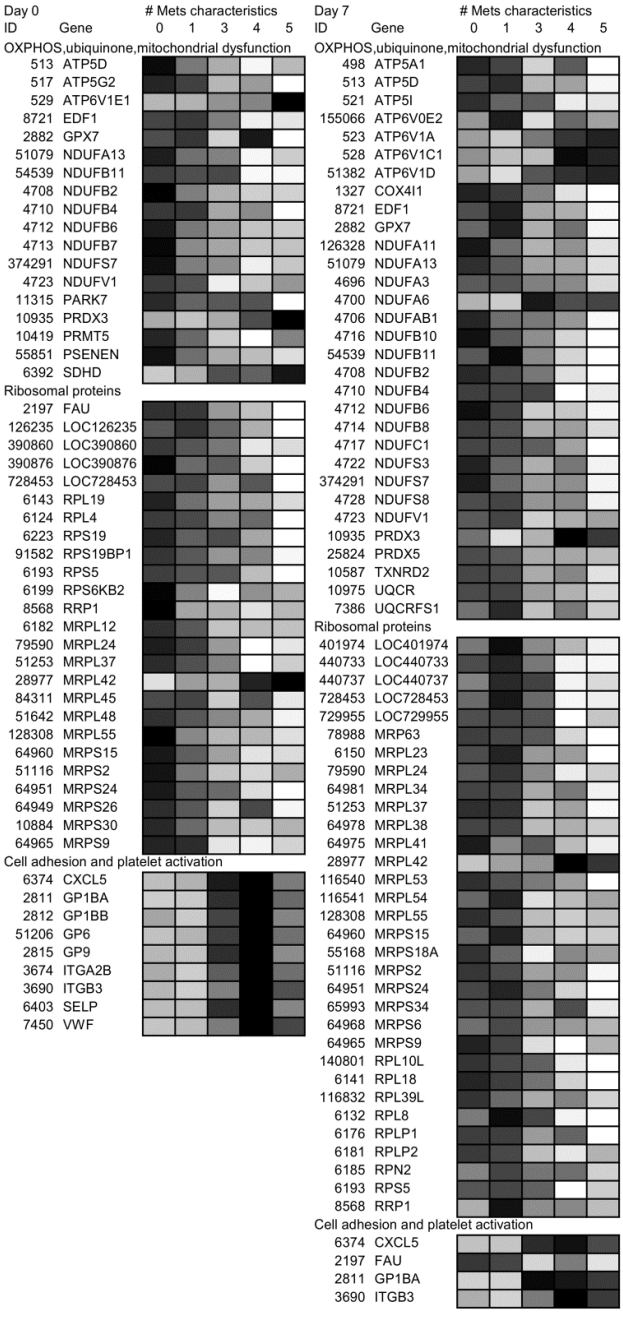
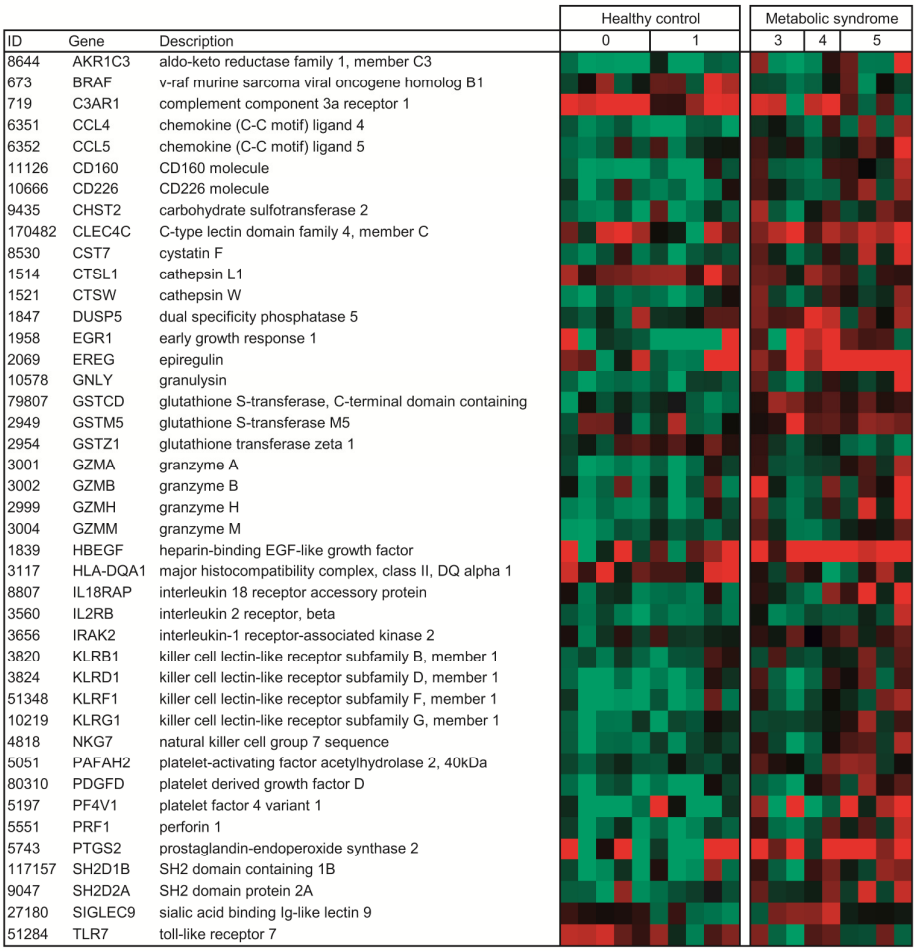

Day 0
Mets characteristics
ID Gene 0 1 3 4 5
OXPHOS,ubiquinone,mitochondrial dysfunction
513 ATP5D
517 ATP5G2
529 ATP6V1E1
8721 EDF1
2882 GPX7
51079 NDUFA13
54539 NDUFB11
4708 NDUFB2
4710 NDUFB4
4712 NDUFB6
4713 NDUFB7
374291 NDUFS7
4723 NDUFV1
11315 PARK7
10935 PRDX3
10419 PRMT5
55851 PSENEN
6392 SDHD
Ribosomal proteins
2197 FAU
126235 LOC126235
390860 LOC390860
390876 LOC390876
728453 LOC728453
6143 RPL19
6124 RPL4
6223 RPS19
91582 RPS19BP1
6193 RPS5
6199 RPS6KB2
8568 RRP1
6182 MRPL12
79590 MRPL24
51253 MRPL37
28977 MRPL42
84311 MRPL45
51642 MRPL48
128308 MRPL55
64960 MRPS15
51116 MRPS2
64951 MRPS24
64949 MRPS26
10884 MRPS30
64965 MRPS9
Cell adhesion and platelet activation
6374 CXCL5
2811 GP1BA
2812 GP1BB
51206 GP6
2815 GP9
3674 ITGA2B
3690 ITGB3
6403 SELP
7450 VWF
Day 7
Mets characteristics
ID Gene 0 1 3 4 5
OXPHOS,ubiquinone,mitochondrial dysfunction
498 ATP5A1
513 ATP5D
521 ATP5I
155066 ATP6V0E2
523 ATP6V1A
528 ATP6V1C1
51382 ATP6V1D
1327 COX4I1
8721 EDF1
2882 GPX7
126328 NDUFA11
51079 NDUFA13
4696 NDUFA3
4700 NDUFA6
4706 NDUFAB1
4716 NDUFB10
54539 NDUFB11
4708 NDUFB2
4710 NDUFB4
4712 NDUFB6
4714 NDUFB8
4717 NDUFC1
4722 NDUFS3
374291 NDUFS7
4728 NDUFS8
4723 NDUFV1
10935 PRDX3
25824 PRDX5
10587 TXNRD2
10975 UQCR
7386 UQCRFS1
Ribosomal proteins
401974 LOC401974
440733 LOC440733
440737 LOC440737
728453 LOC728453
729955 LOC729955
78988 MRP63
6150 MRPL23
79590 MRPL24
64981 MRPL34
51253 MRPL37
64978 MRPL38
64975 MRPL41
28977 MRPL42
116540 MRPL53
116541 MRPL54
128308 MRPL55
64960 MRPS15
55168 MRPS18A
51116 MRPS2
64951 MRPS24
65993 MRPS34
64968 MRPS6
64965 MRPS9
140801 RPL10L
6141 RPL18
116832 RPL39L
6132 RPL8
6176 RPLP1
6181 RPLP2
6185 RPN2
6193 RPS5
8568 RRP1
Cell adhesion and platelet activation
6374 CXCL5
2197 FAU
2811 GP1BA
3690 ITGB3

Figure 3. Average scaled expression levels (scale from -1 white to +1 black) per gene, involved in OXPHOS, ubiquinone and mitochondrial dysfunction, cell adhesion and platelet activation or encoding for (mitochonodrial) ribosomal proteins, for subjects with different numbers of MetS characteristics. Colour scale represents gene expression values relative to the mean of all subjects, with black representing a higher than average expression and white indicating a lower than average expression.

Table 2 Differently changed pathways by CR in MetS subjects and controls

	Ingenuity Canonical Pathways	p value	Genes
1.	Tumoricidal Function of Hepatic Natural Killer Cells	<0.005	*PRF1, GZMB, AIFM1*
2.	Pathogenesis of Multiple Sclerosis	<0.01	*CCL4, CCL5*
3.	Crosstalk between Dendritic Cells and Natural Killer Cells	<0.01	*PRF1, KLRD1, TLR7, CD226, IL2RB*
4.	Aminoacyl-tRNA Biosynthesis	0.021	*RARS, AARSD1, FARSA*
5.	RhoA Signaling	0.021	*ROCK1, SEPT3, CIT, MYL6B, PIP4K2C*
6.	Granzyme B Signaling	0.021	*PRF1, GZMB*
7.	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	0.026	*TLR7, C1QA, CCL5, C3AR1*
8.	Aminosugars Metabolism	0.027	*PDE6G, PGM3, NQO1, LTK*
9.	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	0.027	*CCL4, CCL5*
10.	Graft-versus-Host Disease Signaling	0.028	*PRF1, HLA-DQA1, GZMB*
11.	Gα12/13 Signaling	0.028	*ROCK1, PXN, CDH1, F2R, MYL6B*
12.	Autoimmune Thyroid Disease Signaling	0.030	*PRF1, HLA-DQA1, GZMB*
13.	Granzyme A Signaling	0.030	*GZMA, PRF1*
14.	Actin Cytoskeleton Signaling	0.034	*ROCK1, PXN, F2R, PDGFD, MYL6B, PIP4K2C, MATK*
15.	IL-8 Signaling	0.039	*ROCK1, BRAF, CDH1, HBEGF, PTGS2, IRAK2*
16.	Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	0.039	*CCL4, CCL5*



ID	Gene	Description
8644	AKR1C3	aldo-keto reductase family 1, member C3
673	BRAF	v-raf murine sarcoma viral oncogene homolog B1
719	C3AR1	complement component 3a receptor 1
6351	CCL4	chemokine (C-C motif) ligand 4
6352	CCL5	chemokine (C-C motif) ligand 5
11126	CD160	CD160 molecule
10666	CD226	CD226 molecule
9435	CHST2	carbohydrate sulfotransferase 2
170482	CLEC4C	C-type lectin domain family 4, member C
8530	CST7	cystatin F
1514	CTSL1	cathepsin L1
1521	CTSW	cathepsin W
1847	DUSP5	dual specificity phosphatase 5
1958	EGR1	early growth response 1
2069	EREG	epiregulin
10578	GNLY	granulysin
79807	GSTCD	glutathione S-transferase, C-terminal domain containing
2949	GSTM5	glutathione S-transferase M5
2954	GSTZ1	glutathione transferase zeta 1
3001	GZMA	granzyme A
3002	GZMB	granzyme B
2999	GZMH	granzyme H
3004	GZMM	granzyme M
1839	HBEGF	heparin-binding EGF-like growth factor
3117	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1
8807	IL18RAP	interleukin 18 receptor accessory protein
3560	IL2RB	interleukin 2 receptor, beta
3656	IRAK2	interleukin-1 receptor-associated kinase 2
3820	KLRB1	killer cell lectin-like receptor subfamily B, member 1
3824	KLRD1	killer cell lectin-like receptor subfamily D, member 1
51348	KLRF1	killer cell lectin-like receptor subfamily F, member 1
10219	KLRG1	killer cell lectin-like receptor subfamily G, member 1
4818	NKG7	natural killer cell group 7 sequence
5051	PAFAH2	platelet-activating factor acetylhydrolase 2, 40kDa
80310	PDGFD	platelet derived growth factor D
5197	PF4V1	platelet factor 4 variant 1
5551	PRF1	perforin 1
5743	PTGS2	prostaglandin-endoperoxide synthase 2
117157	SH2D1B	SH2 domain containing 1B
9047	SH2D2A	SH2 domain protein 2A
27180	SIGLEC9	sialic acid binding Ig-like lectin 9
51284	TLR7	toll-like receptor 7

Figure 4 Selection of genes differentially changed by CR in healthy control and MetS subjects. Individual expression changes (signal log ratios) are shown on a color scale (-0.5 is green, 0.5 is red).

DISCUSSION

This study demonstrated that PBMC gene expression profiles are different between female MetS and healthy control subjects and that the differences between these subject groups became more pronounced after an extreme CR challenge. Moreover, we showed that a CR challenge had distinct effects in MetS and healthy subjects.

Several genes involved in pathways related to mitochondrial energy production (i.e. OXPHOS, ubiquinone biosynthesis, mitochondrial dysfunction) were lower expressed in MetS subjects compared to healthy subjects. One of the major regulators of mitochondrial energy production, *PGC1α*, was also lower expressed in MetS subjects. Moreover, a number of mitochondrial ribosomal proteins (MRPs) were lower expressed in MetS subjects. MRPs are needed for the translation of mRNAs encoding for components of the OXPHOS system. The combination of lower expression of *PGC1α,* several mitochondrial energy production genes and a lower expression of MRPs in MetS subjects can point to mitochondrial dysfunction resulting in impairments in mitochondrial energy production [227].These finding support the prevailing hypothesis that defective mitochondrial function is central to chronic diseases such as obesity and type 2 diabetes [228]. The presence of insulin resistance and diabetes have been associated with lower mitochondrial mass, reduced expression of genes related to mitochondrial energy production and reduced expression of ribosomal proteins in PBMCs and muscle [134, 135, 229, 230]. This has led to the hypothesis that mitochondrial deficiency would cause insulin resistance, however results from other studies invalidated this hypothesis [136, 231, 232]. Chronic over nutrition or high-fat consumption leading to obesity have been considered as potential causes of mitochondrial dysfunction [137]. On the other hand, it was shown that obese subjects who were not responding to a weight loss intervention had lower expression of OXPHOS genes in whole blood than obese subjects who were losing weight during the intervention [233]. Moreover, young, non-insulin resistant subjects that are susceptible to weight gain on a high-fat diet showed lower expression of OXPHOS genes in adipose tissue compared to lean subjects that were non-susceptible to weight gain [234]. These results might suggest that diminished mitochondrial functioning is a not a consequence but merely a cause of obesity.

In PBMCs of MetS subjects we also measured a higher expression of genes involved in immune signaling processes, possibly pointing to a higher pro-inflammatory status of MetS subjects. Furthermore, genes involved in cell adhesion and platelet activation such as several glycoproteins, *vWF* and adhesion molecule P selectin were lowered expressed in MetS subjects compared to control healthy subjects. It has been reported that circulating plasma glycoproteins Ib and IX levels are positively associated with

metabolic syndrome [235]. Moreover, it is known that platelets of diabetic subjects show increased surface expression of glycoproteins *GP1b* and *GPIIb/IIIa* [236, 237] and increased expression of adhesion molecules [238]. The higher expression of these genes in our study was measured in PBMCs and not in platelets, but the gene expression changes in the PBMCs may reflect systemic changes and could be indicative of a pro-atherogenic state of MetS subjects.

Since we collected PBMC samples from the same subjects both before and after CR we were able to see which differences between MetS and healthy subjects were consistent over time. More importantly we were able to determine the effects of the extreme CR challenge in MetS and healthy subjects. Since CR is often applied for its positive effects on health and rapid weight loss one might expect that gene expression profiles of MetS subjects after CR would become more similar to those of healthy subjects, as was previously seen for adipose tissue gene expression profiles after a longer CR intervention [74]. Interestingly, we observed that the total number of differently expressed genes between MetS and healthy subjects was increased after CR. In general the CR challenge magnified existing differences in gene expression profiles, as seen for expression of genes encoding for ribosomal proteins and genes involved in mitochondrial energy and pyrimidine metabolism. Pyrimidine metabolism is coupled to the mitochondrial respiratory chain via the mitochondrial enzyme dehydroorotic acid dehydrogenase (DHODH) which was also lower expressed in MetS subjects (borderline significant). It has been suggested that mitochondrial dysfunction could result in a decrease in pyrimidine synthesis. The lower expression of genes involved in mitochondrial dysfunction in MetS subjects might thus possibly be linked to the lower expression of genes involved in pyrimidine metabolism [239].

Beneficial health effects of CR in other studies were mainly reported on the long term, when CR is relatively mild. It is arguable whether it is beneficial for MetS subjects to follow the extreme regime of CR applied in our study. We hypothesize that regular exposure to (nutritional) challenges may improve the metabolic condition which might on the long run result in an increased ability to handle stressful metabolic situations [240]. However, on the short term the CR challenge did not only magnify the differences between MetS and control subjects but did also induce different responses in MetS and healthy subjects, for instance for immune genes. We observed that CR downregulated genes involved in NK-cell immune response, but only in healthy subjects. NK-cell activity was earlier found to be affected by fasting and the number of perforin, granulysin, and/or granzymes A/B-expressing cells has been associated with eating a balanced diet [241, 242]. A change in expression of genes involved in NK-cell response after an alteration in nutritional status is therefore probably a normal response of a healthy body, but this response was not seen in MetS subjects. Whether this should

be interpreted as an impaired response of MetS subjects to nutritional challenges or as an indication of sustained immune functioning of MetS subjects after CR is questionable. In MetS subjects and not in healthy subjects, the genes *EREG, EGR1* and *PTGS2* were upregulated after CR which might point to a pro-inflammatory response in MetS subjects.

Previous studies have shown that CR can reduce weight with concurrent downregulation of oxidative stress and inflammation genes in PBMCs of obese subjects and upregulation of genes involved in mitochondrial biogenesis in muscle [243] [128, 244]. These effects on gene expression were not seen in PBMC from the MetS subjects in our study, despite their weight loss. This might be due to the higher degree of CR applied in our study compared to the other studies.

A limitation of our study was the heterogeneity within the MetS subject group. MetS is defined as a combination of 3 to 5 metabolic risk factors. Based on this definition subjects with different combinations of these characteristics could be included in the MetS group. The health status and response of subjects to a challenge might vary and depend on the specific characteristics present. Interestingly, we observed that an increasing number of characteristics, regardless which characteristics, resulted in lower expression of genes involved in mitochondrial energy metabolism. We should also take into consideration that PBMCs are a mixture of cell populations and therefore changes in PBMC gene expression, such as the changes in genes related to NK-cell response, could also be due to a shift in cell populations. It was previously shown that fasting can decrease the total number of lymphocytes and the percentage of CD4+ cells but other lymphocyte subsets (CD2, CD3, CD8 and CD19) were not changed [241]. We did not determine changes in cell populations and can therefore not exclude whether the observed gene expression changes were due to changes in cell populations.

One of the strengths of this study is the second measurement in the same individuals after CR which reflects similar differences before CR and showed that differences become more pronounced after CR. This points to quite consistent, although small, differences between healthy control subjects and MetS subjects and shows the reproducibility of PBMC gene expression measurements. Our study highlighted the applicability of PBMCs as a model to reflect metabolic disease state and to study the systemic effects of MetS.

In conclusion, we have shown that an extreme CR challenge enlarges differences in PBMC gene expression between MetS subjects and healthy subjects. The differences in PBMC gene expression response to CR in both groups suggests that metabolic status can play an important role in adaptive molecular responses to metabolic challenges.

SUPPLEMENTAL DATA

Supplemental table 1 Selection of significantly different expressed genes between MetS and healthy control subjects

Gene symbol	Gene name	Before CR FC MetS/controls	After CR FC MetS/controls
Cell adhesion and platelet activation			
GP1BA	glycoprotein Ib (platelet), alpha polypeptide	1.66	1.58
GP1BB	glycoprotein Ib (platelet), beta polypeptide	1.56	n.c.
GP6	glycoprotein VI (platelet)	1.28	n.c.
GP9	glycoprotein IX (platelet)	1.48	n.c.
ITGA2B	integrin, alpha 2b	1.71	n.c.
ITGB3	integrin, beta 3	1.47	1.41
CXCL5	chemokine (C-X-C motif) ligand 5	1.90	1.93
SELP	selectin P	1.58	n.c.
VWF	von Willebrand factor	1.26	n.c.
Ribosomal proteins			
LOC126235	similar to 40S ribosomal protein S4, X isoform	-1.09	n.c.
LOC390860	similar to 60S acidic ribosomal protein P0 (L10E)	-1.15	n.c.
LOC390876	similar to 60S ribosomal protein L35	-1.14	n.c.
LOC728453	similar to 40S ribosomal protein S28	-1.10	n.c.
RPL19	ribosomal protein L19	-1.05	n.c.
RPL4	ribosomal protein L4	-1.06	n.c.
RPS19	ribosomal protein S19	-1.05	n.c.
RPS19BP1	ribosomal protein S19 binding protein 1	-1.05	n.c.
RPS5	ribosomal protein S5	-1.09	-1.06
RPS6KB2	ribosomal protein S6 kinase, 70kDa, polypeptide 2	-1.07	n.c.
RRP1	ribosomal RNA processing 1 homolog	-1.09	-1.06
FAU	40S ribosomal protein S30	-1.04	-1.06
LOC401974	similar to ribosomal protein S2	n.c.	-1.08
LOC440733	similar to 40S ribosomal protein S15	n.c.	-1.15
LOC440737	similar to 60S ribosomal protein L35	n.c.	-1.10
LOC728453	similar to 40S ribosomal protein S28	n.c.	-1.11
LOC729955	similar to ribosomal protein L18a	n.c.	-1.08
RPL10L	ribosomal protein L10-like	n.c.	-1.11
RPL18	ribosomal protein L18	n.c.	-1.08
RPL39L	ribosomal protein L39-like	n.c.	-1.17
RPL8	ribosomal protein L8	n.c.	-1.05
RPLP1	ribosomal protein, large, P1	n.c.	-1.04
RPLP2	ribosomal protein, large, P2	n.c.	-1.04
MRP63	mitochondrial ribosomal protein 63	n.c.	-1.07
MRPL12	mitochondrial ribosomal protein L12	-1.11	n.c.
MRPL23	mitochondrial ribosomal protein L23	n.c.	-1.15
MRPL24	mitochondrial ribosomal protein L24	-1.10	-1.06
MRPL34	mitochondrial ribosomal protein L34	n.c.	-1.13
MRPL37	mitochondrial ribosomal protein L37	-1.11	-1.16
MRPL38	mitochondrial ribosomal protein L38	n.c.	-1.10
MRPL41	mitochondrial ribosomal protein L41	n.c.	-1.07
MRPL42	mitochondrial ribosomal protein L42	1.18	1.15
MRPL45	mitochondrial ribosomal protein L45	-1.06	n.c.
MRPL48	mitochondrial ribosomal protein L48	-1.09	n.c.
MRPL53	mitochondrial ribosomal protein L53	n.c.	-1.14

MRPL54	mitochondrial ribosomal protein L54	n.c.	-1.16
MRPL55	mitochondrial ribosomal protein L55	-1.11	-1.12
MRPS15	mitochondrial ribosomal protein S15	-1.11	-1.11
MRPS2	mitochondrial ribosomal protein S2	-1.08	-1.11
MRPS18A	mitochondrial ribosomal protein S18A	n.c.	-1.07
MRPS24	mitochondrial ribosomal protein S24	-1.08	-1.11
MRPS26	mitochondrial ribosomal protein S26	-1.07	n.c.
MRPS30	mitochondrial ribosomal protein S30	-1.07	n.c.
MRPS34	mitochondrial ribosomal protein S34	n.c.	-1.06
MRPS6	mitochondrial ribosomal protein S6	n.c.	-1.06
MRPS9	mitochondrial ribosomal protein S9	-1.15	-1.16
Other			
DHODH	dihydroorotate dehydrogenase	n.c.	-1.09*
PGC1α	peroxisome proliferator-activated receptor gamma,	-1.22	-1.24

Not significantly changed, p value<0.05 (n.c.). A positive fold change (FC) indicates that the gene is higher expressed in MetS subjects compared to control subjects, a negative FC indicates that the genes is lower expressed in MetS subjects compared to control subjects. *borderline significant (p=0.06)

CHAPTER 7

General discussion

In this thesis we investigated the acute and longer-term effects of intake of different types of dietary fat. In addition we identified plasma protein profiles that were associated with BMI and insulin concentrations of abdominally overweight subjects. Furthermore, we investigated how subjects with different metabolic risk phenotypes respond to dietary challenges, such as a high-fat load varying in fat type or a period of extreme caloric restriction.
In this chapter both local and systemic effects of intake of different types of fat will be discussed, followed by a discussion of the use of protein profiling and challenge test for the determination of health status. In addition to the results of our studies, several relevant methodological issues will be outlined and our findings will be discussed in relation to current dietary recommendations.

Local effects of fatty acids in adipose tissue
We showed that consumption of a high SFA diet for 8-weeks resulted in increased expression of genes involved in inflammation processes in adipose tissue whereas consumption of a MUFA-rich diet led to a more anti-inflammatory gene expression profile. These findings are of specific interest since inflammation in adipose tissue has been associated with insulin resistance and plays a role in the onset of T2DM and metabolic syndrome [40, 64, 245]. Changes in inflammatory status have mainly been measured in parallel with changes in adipose tissue mass [50, 51] but according to our results eating a diet with 20 energy% SFA for 8 weeks, without weight gain, may also induce inflammation in adipose tissue.
The effect of the SFA diet on inflammation genes may be mediated via different mechanisms. Genes involved in T-cell and B-cell related processes and chemokines were significantly upregulated after SFA consumption which may point to increased attraction and activation of immune cells in adipose tissue. Moreover, we already suggested that increased activation of the Toll-like Receptor pathway or decreased activation of PPARγ activation may play a role in the SFA-mediated effects on inflammation. The reduced PPARγ expression after the SFA diet, accompanied by decreased expression of adiponectin and increased expression of CD11c and EMR1(F4/80) might also point to a polarisation from alternatively activated macrophages (M2) towards more classically activated pro-inflammatory macrophages (M1), a phenotypic switch which is also associated with obesity-induced adipose tissue inflammation [245-248].
Recently, activation of caspase 1 by a protein complex called the inflammasome has been identified as a novel pathway that can play a role in obesity-induced adipose tissue inflammation and insulin sensitivity [249, 250]. Consumption of the SFA-rich diet did not change gene expression of caspase 1 itself, but upregulated expression of

some genes involved in its activation such as ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), NOD2 (nucleotide-binding oligomerization domain containing 2) and AIM2 (absent in melanoma 2). Activation of caspase 1 can lead to processing of IL1β and IL18, and although expression and plasma protein concentration of IL1β was unchanged, gene expression of IL18 was increased after the SFA-rich diet. Expression of these inflammasome-related genes were unchanged by the MUFA diet which is in line with recent findings of Wen et al. showing that the specifically SFA, but not MUFA, induces activation of the NLRP3-ASC inflammasome in mouse macrophages [251]. Overall, our results suggest that inflammasome-mediated caspase 1 activation might be involved in SFA diet-induced inflammation, but these findings need to verified before clear conclusions could be drawn.

Our study also showed that consumption of a MUFA-rich diet may have anti-inflammatory effects in adipose tissue since it downregulated macrophage genes and genes of the complement system. The complement system plays an important role in initiation of inflammatory processes. Our results in adipose tissue and our findings of a positive correlation of plasma complement 3 concentration with BMI and insulin concentration also suggest a potential role of the complement system in adipose tissue inflammation and insulin resistance [171].

The observed gene expression changes on inflammatory genes in adipose tissue after the SFA-rich and the MUFA-rich diet occurred in the absence of clear systemic effects on whole body insulin sensitivity, on circulating pro-inflammatory plasma proteins or on pro-inflammatory gene expression in circulating PBMCs. Therefore we hypothesize that the observed adipose tissue gene expression changes are early diet effects that are only locally manifested but may later lead to more systemic effects.

Studies in mice revealed that T-cell and B-cell infiltration are the first steps in adipose tissue inflammation and development of obesity-mediated insulin resistance, followed by accumulation of macrophages in a later stage when also insulin resistance develops [34] [252, 253]. Our findings that an SFA-rich diet induced changes in expression of genes involved in T-cell and B-cell related processes in adipose tissue without changes in insulin sensitivity or macrophage infiltration are in line with these findings and support our hypothesis that the gene expression changes in adipose tissue are early diet effects. In humans little is known about the sequence of events in adipose tissue inflammation tha may lead to insulin resistance. One human overfeeding study reported that weight-gain induced insulin resistance was observed in the absence of changes in inflammation in adipose tissue, thereby suggesting that local inflammation occurs secondary to insulin resistance in humans [254]. This which would contradict

our hypothesis, but weight gain-induced effects might be different from diet-induced effects without weight gain. Moreover, only expression changes of macrophage-related genes were measured in adipose tissue samples of the overfeeding study so changes in T-cell and B-cell related genes and pathways may not have been detected.

In our study no effect of the SFA-rich or MUFA-rich diet was observed on insulin sensitivity, neither when determined by euglycemic hyperinsulinemic clamp in a subgroup of subjects from which adipose tissue samples were collected, nor when determined by HOMA-IR in the total study population [255]. Our initial hypothesis that replacement of SFA by MUFA would differently affect insulin sensitivity was based on epidemiological evidence and results from intervention studies at that time which suggested a positive effect of MUFA on insulin sensitivity [6, 16, 17, 256, 257]. The low number of subjects and the relatively short duration of our diet intervention might be possible explanations for not finding an effect on insulin sensitivity in our study. However, two recently performed multi-centre studies investigating the effect of fat modification on insulin sensitivity for longer periods in a higher number of subjects also showed no effect of replacement of SFA by MUFA on insulin sensitivity [18, 19]. Based on these results it is questionable whether there is indeed an effect of replacement of SFA by MUFA on insulin sensitivity.

Obesity-induced adipose tissue inflammation has often been associated with increased concentrations of pro-inflammatory proteins (adipokines) [36] [258]. Although a small decrease of plasma adiponectin concentration was measured in the SFA diet group, in general our intervention diets did not induce clear differential changes in concentration of circulating pro-inflammatory proteins, except for changes in ApoB, CTGF, myoglobin, IL12p70, SHBG and TBG concentrations that decreased with MUFA compared to SFA, but these proteins are not well-known to be secreted from adipose tissue. Other tissues can also secrete proteins and therefore plasma levels may reflect more systemic changes and not necessarily the degree of inflammation within one specific tissue. The absence of changes in pro-inflammatory proteins might thus be because the inflammation in adipose tissue was still a local effect, not resulting in release of pro-inflammatory adipokines, or because changes in adipokine concentrations after 8 weeks were still too small to detect in plasma, possibly due to the contribution of other non-inflamed tissues.

In our study no systemic effects of the diets on PBMC gene expression of inflammatory genes were measured. Previous studies have shown that intake of various types of fatty acids or diets had differential effects on PBMC gene expression related to inflammation [24, 59, 125, 259]. Also in our high-fat challenge study we observed distinct effects of different fatty acids on expression of inflammatory genes in PBMCs, but hardly any effects on inflammatory genes were seen in our longer-term intervention

study. PBMCs travel through the whole body and are exposed to all factors present in the circulation and therefore gene expression changes in these cells, in comparison with adipose tissue, may reflect more systemic effects induced by consumption of the different diets. The absence of a diet-induced effect on inflammation in PBMCs might thus support our hypothesis that consumption of the intervention diets for 8 weeks only had a local effect in the adipose tissue, before systemic effects could be detected. However, there might also be other reasons for the absence of a diet effect on inflammation in PBMCs. In the first place, the different sampling moment of the blood and adipose tissue samples may have affected the results. Due to practical limitations, most adipose tissue samples were collected before the run-in period of the study, when subjects were still consuming their regular diet, whereas PBMCs were collected after 2 weeks run-in on a SFA-rich diet. For PBMCs, the effects of consumption of a SFA diet were thus measured when subjects were already adapted to this diet for 2 weeks, which might explain why fewer changes were found in PBMCs compared to adipose tissue.
Moreover, the unexpected findings of an extremely high inflammatory gene expression in some PBMC samples, possibly due to contamination during the sample processing, might have overruled small diet-induced effects on inflammation.

In summary, it was seen that a SFA rich diet induced a pro-inflammatory gene expression profile in adipose tissue, whereas a MUFA rich diet induced a more anti-inflammatory profile. The fact that these effects were not accompanied by systemic effects on insulin sensitivity, circulating pro-inflammatory proteins or pro-inflammatory gene expression in PBMCs might be due to various reasons as outlined above, but in our opinion the most plausible explanation for our findings is that the effects of the diets after 8 weeks are still local. Adipose tissue could be an early response organ for dietary fat-induced changes and the changes in pro-inflammatory gene expression might be one of the first hallmarks in the development of adipose tissue inflammation and insulin resistance.

Systemic effects of fatty acids in blood

In both the longer-term intervention study as well as the high-fat challenge study the effects of intake of different fatty acids on PBMCs gene expression were measured. In the longer-term intervention study it was shown that MUFA intake lowered expression of OXPHOS genes in PBMCs, in both a western type diet and a MED type of diet, which thus might be regarded as a specific MUFA effect. The effect of MUFA on lowering expression of OXPHOS genes might seem a little unexpected in light of the findings from our caloric restriction study showing that lowered expression of OXPHOS genes in PBMCs is associated with metabolic syndrome. We hypothesize

that MUFA intake increased metabolic health and thereby reduced metabolic stress and OXPHOS activity. The lowered OXPHOS gene expression after MUFA intake might thus be due to a reduced need for OXPHOS capacity while the lowered expression in metabolic syndrome subjects might reflect reduced OXPHOS capacity due to mitochondrial dysfunction. Whether this reduced OXPHOS in MetS subjects is a cause or a consequence of insulin resistance and/or obesity has been subject of debate in several studies [136, 260, 261], but based on our results we cannot draw clear conclusions about this cause-effect relationship.

PBMCs were also used to investigate the response to high-fat challenges containing SFA, MUFA, or n-3 PUFA in subjects with different metabolic risk phenotypes. This challenge study identified a high MUFA load as the most challenging for the biological system, based on the metabolic response and the PBMC gene expression response. The high MUFA load had the highest TAG-raising effect compared with SFA and n-3 PUFA. Since postprandial TAG levels are associated with CVD risk [198], one might conclude based on these results that MUFA intake is pro-atherogenic, which would be in contrast to our previous conclusions and results from earlier studies, showing a LDL-lowering effect of MUFA intake which would suggest an anti-atherogenic effect of MUFA [14, 255]. We hypothesize that consumption of an acute high dose of MUFA in the form of oleic acid (±70 en%) would pose a higher stress to the metabolic system compared to gradual adaptation to a diet containing 20 en% of MUFA in combination with a significant amount of other fatty acids and other macronutrients. Results from the studies of Bouwens et al. illustrate this hypothesis by showing that acute high n-3 PUFA intake caused a stress response in gene expression while long term low n-3 PUFA intake had anti-inflammatory gene expression effects in PBMCs [24] [59]. However, for SFA intake we observed opposite outcomes; longer-term consumption of SFA was found to be pro-inflammatory, whereas an acute high SFA load did elicit a lower metabolic and immune cell response compared to a high MUFA load. This may be because the SFA load, high in palm oil, contained a mixture of fatty acids which is more comparable with normal dietary fat composition. Therefore it could be a less strong challenge for the metabolic system than a high dose of one specific fatty acid, in the case of the MUFA load.
In summary, our results clearly showed that acute effects of fatty acids can be very different from longer term effects of the same type of fatty acids. Acute consumption of an extremely high dose of one specific fatty acid, which is not regularly consumed, might be a challenge for the metabolic system, whereas longer term consumption of a lower dose of the same fatty acid can have positive health effects.

Defining metabolic health status

For defining health status in nutrition research often traditional medical biomarkers were used but these are mainly markers for disease and do give limited information about health status. A multimarker approach to measure protein profiles and gene expression profiles instead of the traditional single marker approach might result in better phenotyping and improved detection of small deviations from a healthy phenotype. Results from our diet intervention study already showed that by measuring a whole genome expression profile subtle, early diet-induced changes could be detected and changes in processes could be identified because changed genes clustered together in pathways with similar function. The value of measuring protein profiles for health status was also illustrated in this thesis. Protein profiling was used to define clusters of plasma proteins that are associated with BMI and insulin in healthy populations. Concentrations of single proteins were not highly elevated in individual subjects, but by comparing protein profiles, subjects which might be more likely to have increased risk for obesity-related disease may be identified. We also revealed that proteins such as MCSF, SAP, IL13 and IL3 were higher correlated with BMI and insulin than well-known and often measured biomarkers for obesity-related disease risk such as IL6 and TNFα. The plasma concentration of some of the identified proteins such as IL3 is very low in non-diseased subjects, but despite that we were able to show a correlation with BMI in two small populations, however, the practical use of IL3 as a biomarker of health status would require sensitive measurements. Due to the explorative character of this study no clear conclusions could be drawn about the predictive value of the identified protein clusters for disease risk and therefore it would be interesting for future research to measure the identified protein clusters in prospective cohort studies.

Another method we have used for more accurately defining health status was to impose subjects to nutritional challenges such as a high-fat load or a period of extreme caloric restriction (CR). Both the high-fat challenge study and the CR study showed the potential of using challenges tests to reveal differences between subjects with different metabolic phenotypes. The different responses to a challenge of obese, obese diabetic and metabolic syndrome subjects compared to lean healthy subjects indicates that the presence of a metabolic risk phenotype changes subjects' ability to handle stressful metabolic situations.

The results from the CR study clearly showed that differences in PBMC gene expression between metabolic syndrome and control subjects were enlarged by a CR challenge. This is a good example how differences in health status become more pronounced after a challenge and illustrates the relevance of a challenge test in defining

small differences in health status between subjects that could not be detected in the 'unchallenged' condition. Although an one week CR challenge might not be the most practical to perform, high-fat challenges, glucose tolerance tests or other types of acute challenge tests might be incorporated in intervention studies to test changes in different aspects of health status [61, 62] [200].
In our studies we have compared responses to challenges between subject groups that were already pre-selected based on differences in BMI or health status. Future studies are needed to determine whether challenge tests could also reveal differences between subjects with more similar phenotypes.

Methodological issues
In addition to the results of our studies there are several methodological issues concerning the design of the studies, the high between-subject variation and small effect size and the intervention diets/shakes that need to be discussed.
One difficulty in human studies is the high variation in responses in gene expression and protein concentrations between subjects. People are metabolically, physiologically, and genetically different which provides interesting possibilities for dietary advices according to person's genetic profiles, but it makes it difficult to define robust effects of a nutritional intervention. Effects caused by other factors than the intended nutritional effects should therefore be minimized, which requires good study designs and careful selection of subjects. In the high-fat challenge study we therefore used a cross-over design that allowed within subject comparison. Although this is the most optimal design to correct for individual variation in response, using this design for the longer term completely controlled dietary intervention study was practically not feasible. A cross-over design would have tripled the study duration which would be very demanding for the participants, probably resulting in high drop-out rate and/or low adherence to the diets. Therefore a parallel design was used, but gene expression and protein concentrations were measured both before and after intervention to be able to compare within the same subject.
In order to diminish variation between subjects as much as possible certain pre-selection criteria for participation were set. For our longer-term dietary intervention study and the high-fat challenge study subjects were mainly selected based on their age, BMI, glucose tolerance and medication use. However, using these selection criteria might have led to selection of too healthy subjects, not representative for all other overweight or obese subjects of the same age class. There might have been additional selection bias in the dietary intervention study since subjects that were already consuming high olive oil or a Mediterranean type of diet might have been more

willing to participate. This might also be a reason why the SFA diet, compared to the MUFA diet, induced more changes in adipose tissue gene expression.
For the caloric restriction study subjects were selected based on the presence of metabolic syndrome, however, because of the heterogeneity of this syndrome this also resulted in variety within the subject group. To lower this variety and to select a suitable, homogenous target group for a dietary intervention more sensitive and extensive phenotyping of subjects may be required, possibly by applying challenge tests and measuring protein profiles to determine health status, or by using imaging techniques such as MRI.

In general the dietary interventions produced subtle changes in gene expression levels in human tissues, as indicated by relatively low fold changes. Dietary human intervention studies are known for their relatively small effect sizes [55, 262]. Our biological system is quite robust and short term dietary interventions will therefore not induce dramatic changes. As dietary products are freely available and, in contrast to medication, should be consumed without serious adverse side effects and without any instructions about dosage, dietary products are also not expected to induce major changes in gene expression within a short period of time. Although dietary interventions induce small changes in gene expression, expression of a high number of genes can be changed. All these subtle changes may have profound biological effects on the long term but it is a challenge to distinguish noise from data with relevant physiological meaning in large genomic datasets and subsequently to interpret these data and define biological effects.
There is no uniform way of microarray data analysis and selection of the suitable approach depends on several factors [263]. Our method of microarray data analysis has been criticized [264] and in a letter [265] we have extensively addressed these issues but this also deserves some attention in this discussion. In our analysis differently expressed genes were selected based on p value and not on mean fold change since mean fold changes can be highly influenced by a few high responders, especially in small sample sizes when subject variation is high, as in our studies. Moreover, our gene expression data was not corrected for multiple testing which will increase the risk of finding more false positive genes and therefore care should be taken in the analysis of single genes. In our analyses changes in expression of genes were only considered meaningful if several genes were changed in one pathway or network and when genes were changed in the same direction within most subjects. The subtle gene expression changes were often consistent over subjects and pathways, and therefore reflect a clear response of the subjects to the diets. Analysis of data from the caloric restriction study showed that several differences in PBMC gene expression between subject groups

were also consistent over time, showing the reproducibility of PBMC gene expression measurements.

Another important issue in human dietary intervention studies is the selection of the dietary components or diets that are tested, but also the selection of an appropriate control diet is of importance. The SFA diet in our longer-term intervention study was initially assumed to be a control diet which would reflect normal intake of middle-aged people in the Netherlands. However, based on the SFA diet effects in adipose tissue this was certainly not the case for most subjects. The use of the SFA diet as a run-in diet to adjust energy intake and eating habits before the actual intervention started may have affected the study results for parameters measured in blood samples that were collected after run-in. However, because of the highly variable eating habits of subjects the design of a control diet that does not have an effect is impossible. A control diet will always induce changes, not only reflecting effects of participation in a trial or seasonal effects, but also reflecting diet effects.
For our high-fat challenge study we have chosen to compare effects of high-fat shakes with each other and not with controls such as low-fat shakes or water since these will have a different energy and/or macronutrient content, which also can affect the response. Changes after water intake can also reveal fasting or circadian rhythm effects, thereby affecting gene expression [266]. Therefore, comparison between high-fat shakes was in our opinion the best way to test the effects of fat type in a challenge.
In our studies we were interested in the effect of different fatty acids and therefore we used diets and shakes that were very similar in nutrient composition, except for the fat type which would make it easier to attribute the observed diet effects to a specific fat type. However, the fat types were exchanged for each other to keep the total fat content and the energy content similar, which makes it difficult to determine whether the effects we observed in the long-term study are for example the result of an increase in SFA or a reduction in MUFA, or a combination of changes.
We have only tested the effects of 8-weeks consumption of a SFA diet consisting of 50% palmitic acid, 20% stearic acid and 15% myristic acid. Other high SFA diets might have different effects, depending on the chain length of SFA, but also the food in which SFA is present [13] [267]. The same holds true for n-3 PUFA and also for MUFA. In Northern countries MUFA intake is not primarily from olive oil but largely derived from animal products which also contain a significant proportion of SFA and might influence the effect of MUFA. The effects of fatty acids might also depend on the diet in which it is incorporated since many components can interact with each other [108]. This was also a reason why we investigated the effects of MUFA from olive oil in a western type diet and in a Mediterranean (MED) type of diet. However, when

investigating effects of such complex diets it is difficult to establish which effects can be attributed to which components, it might also be the synergy between components. We also hypothesize that it might be the high dietary variety of the MED diet and the exposure to different mild stressing components such as polyphenols, PUFA and alcohol that could have affected gene expression; however, no major differences in stress-related pathways between the diets were found. This might be because we measured longer term MED diet effect in fasting blood samples instead of acute responses to the potential stressing diet components.
A mild degree of daily stress in the form of polyphenols, PUFA or alcohol might stimulate cells to train their response mechanisms which may improve the ability to resist stress and to enhance flexibility [240]. This might also be a mechanism how regular stress to the metabolic system, for instance by metabolic challenges, might on the long run improve the metabolic condition.

Dietary recommendations
Current guidelines emphasize the need to lower SFA intake to reduce risk for coronary heart disease (CHD) which would be in line with consumption of a MED diet or a MUFA-rich diet. Although the effect of reducing SFA on lowering CHD risk are still being discussed [268-270], our results suggest that reducing SFA intake might be beneficial to prevent adipose tissue inflammation, which might in the long run lower the risk for CHD and diabetes.
There is also an on-going discussion which nutrient should best replace SFA. Recent decades the focus of food producers was mainly on reducing the amount of fat by substituting it with carbohydrates. However, replacement of SFA by carbohydrates, especially refined carbohydrates and added sugars, has been associated with increased levels of TAG and reduced levels of HDL [271-273] and adverse effects on glycemic control in diabetic subjects [274, 275].
In general, people prefer to eat fatty foods because these are very palatable and, even though food choice should be more focused on health, still palatability and pleasure are major determinants of food choice. In this regard replacement of SFA by MUFA might be a good alternative since it fulfils the need for palatability and pleasure and our study showed that it was also associated with positive health effects. However, we should be careful with translating effects found on risk markers into effects on disease outcome. On the long term there is still no strong evidence that replacement of SFA by MUFA would have a positive effect on CHD and diabetes risk [267, 269, 270, 276]. Mente et al. concluded in their systematic review that only a Mediterranean dietary pattern, and not MUFA intake, is strongly related to CHD in randomized trials [270]. Although this is one of many studies, including ours, suggesting that adoption of a MED diet pattern

would be beneficial for health [10, 255, 277], there is also criticism on this diet and its applicability. Since there is no universal definition of a MED diet the term can be used to describe different dietary habits in countries around the Mediterranean Sea. Positive health effects of a MED diet as reported in studies around the Mediterranean Sea might be confounded by effects of climate, lifestyle and stress level. However, in the controlled setting of our study also beneficial effects of a MED diet on several CVD risk factors were shown.
Practical issues might withhold people from adopting a MED diet pattern; a MED diet contains many fresh products which need more preparation time and are more expensive compared to the currently consumed energy dense pre-packaged and (semi-) prepared foods. Replacement of SFA by MUFA would be easier to implement in daily diets and also food producers could have a role in this; supermarkets already sell a few products in which SFA is replaced by olive oil, but this could be more widely implemented.

Conclusion

In conclusion, this thesis showed that 8-weeks consumption of a SFA-rich diet resulted in a pro-inflammatory gene expression profile whereas consumption of a MUFA-rich diet caused a more anti-inflammatory profile, in addition to reductions in LDL cholesterol and some plasma proteins. Since the effects of the diets on inflammation were still local and not accompanied by systemic changes in inflammatory status or insulin sensitivity we hypothesize that adipose tissue could be an early response organ for dietary fat-induced changes. The changes in pro-inflammatory gene expression might be one of the first hallmarks in the development of adipose tissue inflammation and insulin resistance which on the longer term may lead to inflammation-related diseases such as metabolic syndrome.
Moreover, our studies showed the potential of whole genome expression profiling, plasma profiling and the use of challenges tests to detect subtle diet effects and small differences in health status. Using these tools in future studies will result in more knowledge about health status and about mechanisms behind dietary effects. Eventually this might lead to earlier detection of small deviations from a healthy phenotype and to evidence-based dietary advice to improve health and to prevent disease.

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SAMENVATTING

Achtergrond

Het aantal mensen met risicofactoren voor het ontwikkelen van type 2 diabetes en hart- en vaatziekten is de laatste jaren hard toegenomen. Het is daarom van belang dat er tijdig met preventie begonnen wordt. Veranderingen in voedingsgewoonten kunnen hierbij een belangrijke rol spelen. Een verandering in type vet, zoals het vervangen van verzadigd vet door onverzadigd vet uit olijfolie, zou een gezondheid bevorderend effect kunnen hebben door de risicofactoren voor ziekte te verlagen. Een Mediterraan voedingspatroon bevat veel olijfolie, maar wordt daarnaast ook gekarakteriseerd door een relatief hoge inname van plantaardige producten (groente, fruit, noten, peulvruchten, graanproducten) en matige inname van vis en rode wijn. Consumptie van dit voedingspatroon wordt geassocieerd met diverse positieve gezondheidseffecten.

Over de onderliggende mechanismes van gezondheidseffecten van voeding is nog niet veel bekend. Om meer kennis te krijgen over deze mechanismes kunnen zogenaamde microarrays gebruikt worden. Dit zijn kleine glasplaatjes waarop fragmenten van duizenden genen van de mens gefixeerd zijn. Genen die actief zijn en tot expressie komen produceren boodschapper-RNA's (mRNA's), die een kopie van de gen-informatie bevatten. Met een microarray wordt gemeten welke genen in een bepaald weefsel tot expressie komen en welke niet. Door microarrays te gebruiken in voedingsonderzoek is te bepalen van welke genen de activiteit verandert wanneer een bepaald type voeding gegeten wordt. Dit geeft aan welke processen er dan in het lichaam veranderd zijn.

Voor het meten van genexpressie zijn lichaamscellen nodig, maar in gezonde mensen zijn niet van alle organen eenvoudig cellen te verkrijgen. Daarom wordt er tegenwoordig ook steeds vaker gekeken naar voedingseffecten in een relatief makkelijk verkrijgbare type witte bloedcellen, de perifere bloed mononucleaire cellen (PBMCs).

Een belangrijk doel van voedingsonderzoek is het voorkomen van ziekte en behoud van gezondheid door middel van goede voeding. Hiervoor is het van belang dat er goede indicatoren van gezondheidstatus zijn zodat vast te stellen is welke mensen een verhoogd risico op ziekte hebben. De huidige indicatoren zijn met name gericht om ziekte op te sporen, maar minder geschikt om gezondheidsstatus nauwkeurig te bepalen. Het gebruik van uitgebreide screenings technieken en het meten van de respons capaciteit van het lichaam op metabool stressvolle situaties zouden mogelijke methodes zijn om een betere indicatie te krijgen van gezondheid.

Doel

Het doel van dit promotieonderzoek was tweezijdig; ten eerste, het bestuderen van acute en langere termijn effecten van consumptie van verschillende types vet. Ten tweede, onderzoeken of een uitgebreide karakterisatie van gezondheidsstatus bereikt kan worden door het gebruik van moderne genomics technieken en door het bepalen van de respons op metabole stress testen.

Methodes

Allereerst is een gecontroleerde voedingsproef uitgevoerd waarbij de effecten van 8 weken consumptie van een voeding rijk aan verzadigd vet en een voeding rijk aan enkelvoudig onverzadigd vet gemeten werden op insulinegevoeligheid, serum cholesterol concentraties en genexpressie in vetweefsel.

Daarnaast zijn ook de effecten van vervanging van verzadigd vet door enkelvoudig verzadigd vet in zowel een 'westers voedingspatroon' als ook in een 'Mediterraan voedingspatroon' vergeleken op genexpressie in PBMCs en plasma eiwit concentraties. Gegevens over plasma eiwit concentraties van de deelnemers, gemeten voordat de voedingsproef begon, zijn gebruikt om eiwitten en eiwitclusters te identificeren die geassocieerd zijn met BMI en plasma insuline concentraties en mogelijk kunnen dienen als indicatoren van gezondheidsstatus. Dezelfde analyses werden gedaan in een 2^{e} groep mensen met overgewicht om bevindingen te verifiëren.

In twee andere studies is onderzocht hoe gezonde mensen met een normaal gewicht en mensen met verschillende metabole risico fenotypen (aanwezigheid van obesitas, en/of diabetes, en/of metabool syndroom) reageerden op metabool stressvolle situaties. In één studie is de metabole en PBMC genexpressie respons op inname van een hoog-vet shake met verschillende types vet vergeleken. In de andere studie is de respons op een periode van extreme restrictie van calorie inname bepaald. In diezelfde studie werden ook de PBMC genexpressie profielen van mensen met metabool syndroom vergeleken met PBMC genexpressie profielen van gezonde mensen.

Resultaten

Resultaten van de gecontroleerde voedingsproef lieten zien dat consumptie van een voeding rijk aan verzadigd vet de expressie van inflammatie- (ontstekings) genen in vetweefsel verhoogde terwijl een voeding rijk aan enkelvoudig onverzadigd vet leidde tot een minder inflammatoir genexpressie profiel. Deze veranderingen gingen niet gepaard met veranderingen in insuline gevoeligheid. Verder leidde een hoge inname van enkelvoudig onverzadigd vet uit olijfolie, als onderdeel van zowel een westerse type voeding als ook van een Mediterrane voeding, tot een verlaging van expressie van genen betrokken bij oxidatieve fosforylering in PBMCs. Daarnaast zorgde hoge

inname van enkelvoudig onverzadigd vet ook voor een verlaging van serum LDL cholesterol en plasma ApoB, connective tissue growth factor en myoglobine concentraties wat kan duiden op een verbeterde metabole gezondheid.
In plasma van de gezonde deelnemers hebben we clusters van eiwitten geïdentificeerd die geassocieerd waren met BMI en insuline waardes. Deze clusters bevatten bekende indicatoren voor obesitas-gerelateerde ziekte en potentiele nieuwe indicatoren.
De studie waarbij de respons op een hoge dosis vet werd gemeten liet zien dat de plasma metabole respons (triglyceriden, insuline, glucose en vrije vetzuren) en de PBMC genexpressie respons na een hoog vet inname beïnvloed werden door de aanwezigheid van obesitas en/of diabetes type 2. Vergelijking van de respons op een hoge dosis verzadigd, enkelvoudig onverzadigd en meervoudig onverzadigd vet liet zien dat een hoge dosis enkelvoudig verzadigd vet de meest uitgesproken respons induceerde, met name in mensen met obesitas en type 2 diabetes, en daarom het meest geschikt lijkt als metabole stress test.
De studie waarbij extreme restrictie van de calorie inname werd toegepast liet zien dat PBMC genexpressie profielen verschillend waren tussen mensen met metabool syndroom en gezonde mensen, met name voor genen die betrokken zijn bij processen gerelateerd aan mitochondriaal energie metabolisme. Verder zagen we dat een periode van extreme calorische restrictie de verschillen in PBMC genexpressie tussen de groepen mensen vergrootte.

Conclusie

Dit promotieonderzoek heeft aangetoond dat 8 weken consumptie van een voeding rijk aan verzadigd vet leidde tot een pro-inflammatoir genexpressie profiel in vetweefsel, terwijl consumptie van een voeding rijk aan enkelvoudig onverzadigd vet leidde tot een meer anti-inflammatoir genexpressie profiel, naast een afname van LDL cholesterol, enkele plasma eiwitten en expressie van oxidatieve fosforyleringsgenen in PBMCs. Omdat de effecten van de voeding op inflammatie vooral lokaal gemeten werden en niet gepaard gingen met systemische veranderingen in inflammatoire status of insulinegevoeligheid komen we tot de hypothese dat vetweefsel mogelijk een vroeg responderend orgaan is voor vet-geïnduceerde veranderingen. De veranderingen in inflammatoire genexpressie zouden één van de eerste tekenen kunnen zijn in de ontwikkeling van systemische inflammatie en insuline resistentie, wat op de langere termijn zou kunnen leiden tot inflammatie-gerelateerde ziektes zoals metabool syndroom.
Onze studies lieten verder zien dat het meten van genexpressie profielen, het meten van plasma eiwit profielen en het gebruik van metabole stress testen geschikte methodes kunnen zijn om subtiele voedingseffecten en kleine verschillen in gezondheidsstatus

aan te tonen. Het gebruik van deze methodes in toekomstige studies kan bijdragen aan meer kennis over gezondheidsstatus en mechanismes achter effecten van voeding. Uiteindelijk zou dit kunnen leiden tot een eerdere detectie van kleine afwijkingen van een gezond fenotype en ‘evidence-based’ voedingsadvies om gezondheid te verbeteren en ziekte te voorkomen.

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CURRICULUM VITAE

Susan van Dijk was born on the 5th of August 1983 in Zutphen, the Netherlands. After completing secondary school at Stedelijk Dalton college in Zutphen in 2001, she started the study 'Nutrition and Health' at Wageningen University and specialized in Food Toxicology and Molecular Nutrition. She did her first Master thesis at the Toxicology Group of Wageningen University on the subject 'The Unscheduled DNA Synthesis test with Liquid Scintillation Counting to test the genotoxicity of methyleugenol derived from basil'. The second Master thesis was done at the Nutrition, Metabolism and Genomics Group where she investigated the role of fasting induced adipose factor. She did her internship at Numico Research, working on in vitro models for reduction of energy status in muscle and liver.

After graduation she started working as a research assistant at the Nutrition Metabolism and Genomics group and in January 2007 she started working on a PhD project financed by the Dutch Diabetes Foundation, investigating the effects of dietary fat in the prevention of type 2 diabetes.

LIST OF PUBLICATIONS

van Dijk SJ, Feskens EJ, Bos MB, Hoelen DW, Heijligenberg R, Bromhaar MG, de Groot LC, de Vries JH, Müller M, Afman LA. A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome. Am J Clin Nutr. 2009 Dec;90(6):1656-64.

Bos MB, de Vries JH, Feskens EJ, **van Dijk SJ**, Hoelen DW, Siebelink E, Heijligenberg R, de Groot LC. Effect of a high monounsaturated fatty acids diet and a Mediterranean diet on serum lipids and insulin sensitivity in adults with mild abdominal obesity. Nutr Metab Cardiovasc Dis. 2010 Oct;20(8):591-8.

van Dijk SJ, Feskens EJM, Heidema AG, Bos MB, van de Rest O, Geleijnse JM, de Groot CPGM, Müller M, Afman LA. Plasma Protein Profiling Reveals Protein Clusters Related to BMI and Insulin Levels in Middle-Aged Overweight Subjects. PLoS One. 2010 Dec 23;5(12):e14422

van Dijk SJ, Feskens EJ, Bos MB, de Groot LC,de Vries JH, Müller M, Afman LA The effects of a diet high in monounsaturated fat and a Mediterranean diet on PBMC whole genome gene expression and plasma proteins. *Submitted for publication*

van Dijk SJ, Mensink MR, Esser D, Feskens EJ, Müller M, Afman LA. Responses to high-fat challenges varying in fat type in subjects with different metabolic risk phenotypes. *Submitted for publication*

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

López S, **van Dijk SJ**, Müller M, Afman LA. Monocytes reflect distinct BMI and fatty acid specific postprandial gene expression changes. *Manuscript in preparation*

Esser D, **van Dijk SJ**, Oosterink E, Müller M, Afman LA. BMI and fatty acid type affect postprandial vascular function and lymphocyte CD11A/CD11B expression. *Manuscript in preparation*

OVERVIEW OF EDUCATIONAL ACTIVITIES

Discipline specific activities
NuGO week 2011 Wageningen
NWO nutrition meetings 2007, 2008, 2009, 2010, Deurne
ISSFAL 2010, Congress of the International Society for the study of fatty acids and lipids, Maastricht
Masterclass Nutrigenomics " Regulation of energy homeostasis", Wageningen, 2009
International conference on Prediabetes and metabolic syndrome, Nice, France 2009
NuGO week 2009 Montecatini Terme, Italy
Nutritional Sciences Forum, Arnhem 2009
International Conference on the Bioscience of lipids, Maastricht 2008
NuGO week 2008 Potsdam, Germany
Meeting Nederlandse Verenging voor Diabetes Onderzoek, Doorwerth, 2007
NuGO week 2007 Oslo, Norway
Hands-on micro array course 2007, Maastricht
Masterclass Nutrigenomics "From molecular nutrition to prevention of disease", Wageningen, 2007

General courses
Career Perspectives, 2011
Writing and presenting a scientific paper, 2010
VLAG introduction week 2007
NuGO introduction course 2007
NuGO days for former introduction course participants, Oslo, 2007

Optionals
PhD tour 2007 USA
Journal club, Division of human nutrition, 2007/2008
Scientific meetings, Nutrition, Metabolism and Genomics Group, 2007-2011
Journal club Nutrition, Metabolism and Genomics Group, 2009/2010
Participation (and organization) PhD retreat, Division of Human Nutrition 2007,2009
Preparing PhD research proposal
Diabetes research club meetings 2009-2010
NuGO Exchange to Marche Polytechnic University Ancona Italy, 2008

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